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Applications and Optimization of Immunization Procedures

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Abstract

Classical immunization protocols have produced an antibody-based humoral response that is very effective against susceptible infectious diseases. Immunization introduces an external substance to induce the host immune system to respond specifically. Typically an antigen is used, but DNA, or a primed, pre-existing leukocyte or antigen-presenting cell, can also be used. Immunization is currently being used or investigated for the prevention and treatment of infectious diseases, cancer, addictions, allergies, pregnancy, and autoimmune diseases. It is also being used to produce biologically active materials such as polyclonal and monoclonal antibodies, antivenins, and anti-toxins for treating a wide range of conditions. Animals have been integral to the development of immunization techniques, as producers of toxoids and antitoxins, as models (e.g., to validate materials and protocols used for immunization, to understand the impact of immunization itself on the immune system, and to help investigators devise methods for determining the efficacy of vaccines) and as beneficiaries themselves of vaccines and antitoxins. The choice of immunization protocols is complex, and results may be affected by many factors such as dose and concentration of antigen, choice of adjuvants, time between inoculation and response measurement, and method of detection. The immune system responses to an antigen are also complex and continue to develop with advancing age. Anatomical, physiological, and immune system differences between species influence responses to immunization, as do the purity and presentation of the antigens and adjuvants. When directly comparing results, animals should be sourced from the same supplier. This review highlights the many uses of immunization techniques and introduces important considerations for the choice of protocols and animal models.

Key Words: adjuvant; animal research; antibody; disease; immunity; immunization; vaccine

Introduction

Immunization has been one of the great technical developments contributing to the freedom from life-threatening infectious diseases that we currently enjoy. Even before the days of Jenner's first vaccine published in 1798 as "An inquiry into the causes and effects of the variolæ vaccinae," inoculation of material from patients with mild cases of smallpox was used to induce a local response and prevent more severe and often fatal systemic disease. We have for many years profited from the benefits of immunization without fully understanding how those benefits occurred. Animals have been integral to the development of immunization techniques, as producers of antitoxins, as models (to validate materials and protocols used for immunization, to understand the impact of immunization on the immune system, and to determine the efficacy of vaccines), and as beneficiaries themselves of vaccines and antitoxins.

Classical immunization protocols produced an antibody-based humoral response that is very effective against susceptible infectious diseases. The ability of the first immunizations to induce protection against viruses, bacteria, and bacterial toxins is the basis of current vaccination protocols. Since then, additional applications of immunization have been used to advance medical science. The targeted impact of immunization procedures in blocking peptide and protein functions has greatly contributed to the understanding of how our immune systems and physiological processes are controlled. This knowledge is being used to adapt new technologies to prevent or control health conditions (e.g., cancers and addictions) that were previously unable to benefit from immunization.

Immunization introduces an external substance to induce the host immune system to respond specifically. Typically this substance is an antigen, and can include DNA or a primed, pre-existing leukocyte or antigen-presenting cell. Currently, immunization is used or is being investigated for the prevention and treatment of an extensive number of conditions ranging from infectious diseases, cancer, addictions, allergies, and pregnancy to autoimmune diseases. It is also being used to produce biologically active materials such as polyclonal and monoclonal antibodies, antivenins, and antitoxins for treating a wide range of conditions. In this article, we highlight the many uses of immunization techniques and discuss important considerations for the choice of protocols and animal models.

Purposes of Immunization

Infectious Disease Prevention

Traditional vaccines have used immunization processes to protect an individual against potential future exposure to an infectious agent. Even when the process is not 100% efficacious, a sufficient proportion of the population is protected and "herd immunity" is created. In such a case, the population is protected against subsequent infections of individuals and endemic persistence of the infectious agent. When an infectious agent infects one individual, another susceptible host is not encountered in the immediate environment and transmission of the organism cannot occur before the transmissible stage of the life cycle of the infection is passed.

A large number of diseases exist for which there is no effective preventive vaccine, usually because the traditional protocol of immunization--with a live attenuated organism, an inactivated organism, or antigenic components of an organism combined with aluminium-based adjuvants--has not been demonstrated to be safe and effective. In many cases, complex immune interactions with the infectious agents may suppress or moderate the host response. In these situations, a simple antibody response against primary surface antigens is not effective. Other strategies used to evade elimination by the host immune system include the following: antigenic variation, latency of infection, ability to replicate intracellularly or within immunoprivileged sites, immune suppression induced through mediators or infection and destruction of crucial immune cells, and integration of viral DNA into the host cell genome. The process of inducing responses against poorly immunogenic antigens has improved in recent years, particularly for polysaccharide antigens such as *Streptococcus pneumoniae* conjugate vaccines, wherein lack of immunogenicity has been overcome by conjugation with larger, more immunogenic molecules (Moreau 1999).

The efficacy of standard vaccines has typically been measured by the degree of humoral response. Protection against disease for which many next-generation vaccines are currently being developed depends on cell-mediated immunity (CMI¹) and specific responses in tissues such as mucosa of the lung, gut, or reproductive tract. Tuberculosis is a typical example for which new protocols add adjuvants to induce type 1 responses, and new routes (e.g., oral vaccination) are used to induce a lung mucosal CMI response by targeting the gut mucosa (Doherty et al. 2002).

Several additional strategies are currently being used to produce vaccines for infections against which traditional vaccines have not been successful. Examples of strategies used to induce effective immunity without adverse consequences include subcomponent vaccines, peptide-based vaccines, recombinant DNA vaccines, chimeric vectors, and alternative routes of administration. These strategies are described further in the respective sections of the text below.

Maternal and Fetal Immunization

Neonates are particularly susceptible to infectious disease and are very dependent on maternal antibodies for protection until they can develop and mount their own immune responses. Neonatal vaccination is complicated by the maternal antibodies and the immaturity of the immune system, but the advent of conjugate vaccines and greater understanding of the immune system are providing opportunities for its application (Marchant and Newport 2001). Routine prophylactic immunization has focused to date on protecting neonates after the maternal antibodies wane. Maternal immunization, in which the dam is immunized against a specific disease to develop high levels of antibodies to pass on to her newborn, has been practiced for many years in veterinary medicine and is being seriously investigated as an approach in human medicine (Lehmann et al. 2003). Placental anatomy and the process of antibody transfer vary greatly between species. Nonhuman primate species have a placental anatomy and physiology most closely linked to humans, which makes them appropriate for this type of research.

The concept of fetal immunization has been demonstrated successfully in various animal models. Fetal immunization with a protein antigen has been studied in baboons, and a single DNA immunization against a truncated form of glycoprotein D of bovine herpesvirus-1 into the amniotic fluid of the oral cavity has resulted in high serum antibody titers and cell-mediated immune response in lambs (Gerdt et al. 2002).

Production of Antitoxin

Immunization has been used to produce antiserum since the 1890s. The antiserum produced is rich in antibodies against the specific antigen inoculated. These processed antisera or extracted immunoglobulins are used to treat life-threatening conditions such as rabies infection, diphtheria, tetanus, botulinum intoxication, and venomous snakebites. Antitoxin and antivenom are generally manufactured from antibody-rich serum produced by horses or other large animals. Multiple booster inoculations of small quantities of toxin or venom are used to induce the production of a hyperimmune response. The booster inoculations produce a large quantity of antibodies that also have a greater affinity for the inciting antigen.

Cancer Treatment

The hypothesis of using vaccines to stimulate the immune system to prevent and treat cancer has been considered for more than a century. In Table 1, the applications of vaccines in cancer therapy are listed. Bacille Calmette-Guérin (BCG¹) is used around the world to treat bladder

cancer (Kassouf and Kamat 2004). Vaccines may also be used to prevent infection by pathogens known to predispose individuals to cancer such as hepatitis B virus or *Chlamydia* spp. (Moingeon 2001).

Table 1 Possible application of vaccines against cancer^a

Modality	Status	Comments
Vaccines (prophylactic or therapeutic against pathogens predisposing to specific cancers)	Ongoing clinical studies—hepatitis B vaccine available	Other targets include oncogenic papilloma viruses, hepatitis C, <i>Helicobacter pylori</i>
Therapeutic (adjuvant setting)	Ongoing clinical trials	Aim is to prevent recurrence after surgical removal
Therapeutic (metastatic disease)	Ongoing clinical trials	Aim is to control and maintain quality of life
Prophylactic	Theoretical	Aim is to prevent high-risk healthy people from developing cancer

^aAdapted from Moingeon P. 2001. Cancer vaccines. *Vaccine* 19:1305-1326.

Several immunological vaccines have been tested in clinical trials for treatment of melanoma. Whole cell vaccines (allogeneic and autologous cellular vaccines) comprise a broad spectrum of antigenic targets. Ganglioside vaccines have been prepared with defined purified antigens that may allow for a specific type of immune response (Guthman et al. 2004). Other types of cancer vaccines under development include DNA vaccines, heat shock protein-based vaccines, peptide vaccines, and dendritic cell vaccines (Wolochok and Livingston 2001).

The majority of current research on cancer vaccines is focused on tumor-associated vaccines. In theory, cancer vaccines should provide a specific immune response against the primary tumor and result in strong immune memory to prevent recurrence. Antigenic differences between normal and malignant cells provide the basis of cancer vaccines to stimulate tumor-specific immune responses. Advances in molecular characterization of tumors have identified tumor-associated antigens that are potential targets for use in cancer immunization protocols (Conroy et al. 1996; Moingeon 2001).

Immunological approaches in cancer vaccines are varied. The normal immune response is not sufficient to eradicate tumor cells, and cancer cells may escape detection via secretion of immunosuppressive factors, down regulation of antigen expression, major histocompatibility complex molecules, or lack of costimulation. Vaccines may be developed that transfer genes of immune-stimulant cells and produce appropriate cytokines or that manipulate antigen-presenting cells such as dendritic cells. Direct injection of immature dendritic cells into tumors is a novel approach being used to induce an immune response based on the processing and presenting of existing antigens from apoptotic cells (Kim et al. 2004a,b). DNA vaccines may also be used after conventional treatments to eliminate metastasis (El-Aneed 2004).

Other Therapeutic Vaccines

Vaccines are under development for a number of chronic infectious and degenerative diseases. Human immunodeficiency virus (HIV¹) is one example. Whole virus, killed virus, and recombinant vaccines have been examined for this purpose but have not shown sufficient efficacy. Vaccines using recombinant live virus vectors appear to have promise, demonstrating both safety and a cytotoxic lymphocyte response (Rocha et al. 2004).

A highly effective vaccine exists that can prevent hepatitis B, and research is now focused on a therapeutic vaccine. Inducing Th1 responses to the hepatitis B core antigen, has been demonstrated to be important in recovery from disease and infection. Peptide vaccines including B cell- and T cell-inducing peptide epitopes have been tested in animal models and clinical trials (Arnon and Ben-Yedidia 2003). Core antigen loaded nanoparticles are also being developed as a strategy to induce an appropriate therapeutic immune response (Chong et al. 2005).

Vaccines are being developed to treat other disease conditions, including tuberculosis, parasitic disease, and gastric ulcers (Arnon and Ben-Yedidia 2003; Sela et al. 2002). Neurodegenerative conditions such as Huntington's and Alzheimer's diseases, in which there is an abnormal accumulation of protein aggregates, are also potential candidates for vaccine treatment (Sela et al. 2002). Nonspecific methods of treating these and other neurodegenerative disorders include using vaccines to boost the aging immune system (Schwartz and Kipnis 2004).

Therapeutic Antibodies

Monoclonal antibodies (MAbs¹) were originally proposed for use in a variety of chronic inflammatory diseases and for preventing transplant organ rejection. MAb therapy has been used to induce immunosuppression and prevent organ rejection. OKT3, the first MAb approved for this indication, inhibits T cell responses by targeted binding of the pan-T cell marker CD3. More recently approved humanized neutralizing MAbs target the interleukin (IL¹)-2 receptor α -chain.

Antibodies protect organisms by binding and neutralizing active molecules, enabling phagocytosis, and activating complement. By capitalizing on some of these functions to inhibit the activity of proinflammatory molecules, they can serve as a useful tool in the potential treatment of chronic inflammatory disease. Antitumor necrosis factor α (TNF α ¹) MAbs have been used in the treatment of rheumatoid

arthritis. Anti-TNF α MAbs developed from a mouse:human chimerized antibody is currently licensed as Inflixmab. Clinical studies have confirmed efficacy with improved clinical responses and arresting of joint degeneration. Studies are also ongoing to investigate anti-IL-1 and IL-6 MAb in rheumatoid arthritis (Andreaskos et al. 2002).

Anti-TNF α therapy has also been studied in other chronic immune and inflammatory conditions such as Crohn's disease, spondyloarthropathies, juvenile arthritis, and psoriasis. Clinical trials have shown promising response in these diseases. Studies investigating the role of antibodies in the treatment and prevention of prion diseases have also shown promise. In vitro studies demonstrate that cultures appear to be rid of the agent, but animal studies are difficult to conduct due to the long incubation of the disease (Sela et al. 2002).

Murine MAbs, although effective, may be poorly tolerated in humans as multidose therapeutics. Chimeric antibody technology followed by humanization may provide antibodies that are still immunogenic. Fully human antibodies are preferred, but to date, there has been limited success in developing human B cell hybridomas. Transgenic mouse technologies have allowed the introduction of transgenes on yeast artificial chromosomes (YACs¹) into the mouse germ-line, generating mice with larger portions of the human immunoglobulin (Ig¹) loci. XenoMouse® contain large base-sized YACs from which IgG MAbs have a diverse human adult-like repertoire with the CDR3 regions more similar in length to human than to mouse (Houdebine 2002; Kellermann and Green 2002).

Diagnostic Reagents

Immunization for antibodies has been used to produce reagents for diagnostic tests that depend on antibodies as part of the detection systems, such as radioimmune assays and enzyme-linked immunosorbent assays (ELISAs¹). Many of the diagnostic tools used to identify proteins or examine the immune system depend on monoclonal or polyclonal antibodies to bind a specific molecule. Detection of the bound complex occurs through light scattering or tagging of the antibody with radioisotopes, fluorescent molecules, or enzymes to elicit a color change.

Treatment of Allergies

Immunization with food proteins at an appropriate level can sensitize animals and cause conditions that mimic human food allergies. One example is a protocol successfully used in dogs to induce peanut and other nut allergies by 6 mo of age. Animals are inoculated s.c. with 1 μ g of in alum, first at birth and then at 3, 7, and 11 wk of age, immediately after modified live virus vaccinations (Teuber et al. 2002). Specific allergen immunotherapy has been effective in treating rhinitis and anaphylaxis. Immunostimulatory DNA has been studied in models of allergen-induced airway inflammation and has shown promising results in mice (Silverman and Drazen 2003; Walker and Zuany-Amorium 2001).

Research Models

As a technique, immunization continues to be used extensively and has benefited from the large number of currently available animal models with well-defined immune cell deficiencies and from the increasing availability of immune modulators such as interleukins. These tools have helped to define more completely how the immune response is controlled. Initially, mice deficient in specific lymphocyte populations (e.g., natural killer cell-deficient mice, nude athymic mice, and severe combined immunodeficient (SCID¹) mice) became available and were widely used. More recently, transgenic mice have provided opportunities to study the immune system in even greater detail. For example, the ability of an adjuvant to activate toll-like receptors (TLRs¹) is derived from a lack of effect on mice deficient in TLR-4.

In addition to the use of immunization techniques to study the immune system itself, immunization is frequently used to block biological reactions using antibodies. This application has been frequent in reproductive research in which neutralizing particular peptides, proteins, or cell surface antigens are used to study reproductive physiology and the etiology of specific diseases.

Autoimmunity and Degenerative Disease Models

Immunization is used to generate models of diseases that have an autoimmune basis. A list of examples is included in Table 2. Many degenerative inflammatory conditions, from diabetes to multiple sclerosis, are understood to have a misdirected immune response that induces pathology. Combinations of immunizations to mimic the conditions and animal models with similar immune alterations are used to understand and develop therapies for these conditions. For example, a systemic lupus erythematosus-like syndrome has been established in mice by inoculation with active chromatin (Li et al. 2004). Multiple sclerosis-like experimental allergic encephalitis has also been established using immunization.

Table 2 Examples of autoimmune degenerative diseases with immunization-induced models

Disease	Model	Animal	Reference (see text)
Multiple sclerosis	Experimental autoimmune encephalitis induced by multiple antigen peptide myelin oligodendrocyte glycoprotein 35-55 (MOG35-55)	C57BL6/J mouse	Costa et al. 2003
Systemic lupus erythematosus	Inoculation with active chromatin	Mouse	Li et al. 2004
Rheumatoid arthritis	Rat adjuvant arthritis	Rat	Ku et al. 1993
Autoimmune disease	Collagen-induced arthritis	Mouse	Chiocchia et al. 1993; Nagler-Anderson et al. 1986
Ulcerative colitis	Carageenan model enhanced by immunization with <i>Bacteroides vulgatus</i>	Guinea pig	Breeling et al. 1988
Interstitial cystitis	Bladder homogenate and complete Freund's adjuvant immunization	Rat	Luber-Narod et al. 1996
Autoimmune uveitis	Interphotoreceptor retinoid binding protein	C57BL/6 mouse	Willbanks et al. 1997
Atherosclerosis	Immunization with heat shock protein 65	Low-density lipoprotein receptor-deficient (LDL-RD) mice	Afek et al. 2000

Immunization protocols are also used to investigate potential therapies. To treat multiple sclerosis-like inflammatory disease in a mouse model, one successful protocol uses autologous attenuated autoreactive T cells to induce an immune response specifically against autoreactive cells in order to attenuate the condition (Stinissen et al. 1996). Beta crystalline autoantibodies can contribute to the development of cataracts. In mice, oral administration of lens homogenate combined with immunization against beta-crystallins in adjuvant has been shown to suppress anti-beta crystalline antibody production (Sueno et al. 1997).

Screening Potential Vaccines and Adjuvants

As part of the initiative to find and introduce new vaccines and new adjuvants, it is necessary to develop methods to determine and optimize the in vivo response to new candidate agents. Bringing a potential agent from discovery to clinical use is an exercise that can take many years and consume many millions of dollars. Screening is the part of the process that can differentiate between many potential antigens, multiple combinations of antigens, and different adjuvant matrices, in an effort to choose those agents with the most potential for success. Immunization protocols in animals are useful for this process because they incorporate the biological complexities of the immune system that may be predictive of the result in the final host as well as being predictive of adverse secondary effects. For example, a foreign peptide that is nonimmunogenic in a mouse may be more likely to be nonimmunogenic in a human. The predictive ability of one animal species for another is not complete. The mouse response to DNA vaccination has been very successful, but such success has not translated as predictably in other mammalian species such as dogs and humans (Kutzler and Weiner 2004).

With an allowance for the constraints of interpretation, animal models have been very useful for screening. Proteins can present with many different antigenic sites. Screening is used to determine which sites will most likely provide the wanted immune response. The human lymphocyte antigen (HLA¹) transgenic mouse expresses human major histocompatibility complex (MHC¹) molecules and is an example of a transgenic mouse strain that has been used for screening a battery of antigen candidates for potential human CMI responses (Firat et al. 1999).

Quality Control/Testing

Animal immunization is required for testing and quality control of vaccines before release to the market. Testing is mandated by regulatory bodies to ensure identity, purity, safety, and efficacy (antigenicity or potency) of each batch of vaccine. The purpose of a potency test is to ascertain that the batch being tested is capable of an adequate biological response and has a potency that reaches a level demonstrated to be protective in humans. The purpose of an antigenicity or immunogenicity test is to compare consistency of manufacturing, using antibody levels as a measure that each batch contains the same amount of immunogen as the standard batch. Alternative methods for more precisely measuring the biochemical quality or purity of antigens avoid the need for an in vivo immunization protocol for some vaccines. For many older vaccines, the precise immunogens or biochemical determinants of potency have not been isolated.

Unlike typical immunization protocols that use a prime-boost strategy, the protocol for immunogenicity testing is often limited to a single dose because the first response is most capable of discriminating the amount and quality of the immunogen. A booster response is influenced by other factors, and the results can be less discriminating in detecting differences between two different vaccine lots. In all cases, it is important to have supporting data to establish the appropriate animal species, method of analysis, and specifications. For traditional vaccines, clearly defined testing protocols are often established by federal licensing agencies. For new vaccines, appropriate in vivo and in vitro tests must be established as a critical part of the development plan. These tests are often linked with the clinical efficacy trials.

A typical vaccine potency test measures the level of protection either against a direct challenge, using a known quantity of infectious organisms, or through an indirect step, such as exposing toxin to neutralizing serum before challenge. The determination of potency in these cases is generally made through a series of dilutions that are compared with dilutions of a standard reference prepared under the same conditions. Based on this comparison, a quantitative result can be assigned in accordance with units assigned to the reference vaccine. Once sufficient experience is obtained to demonstrate the consistency of the production and testing systems, it is possible to move to a single dilution test that demonstrates the consistent achievement of a minimum value (Akkermans et al. 1996). This process limits the number of animals required.

Some challenge protection test methods (e.g., the mouse pertussis intracerebral challenge test, also known as the Kendrick test) remain in use because they have been linked to field or clinical trial data (Standfast 1958). As a refinement of these challenge potency tests, *in vitro* methods are being introduced to assess antibody levels by serological assays such as ELISA or toxin neutralization assays that have demonstrated correlation with the potency tests. Many regulatory jurisdictions are beginning to accept this refinement for tetanus and diphtheria testing (Sesardic et al. 1999).

Tests using immunizations are also conducted to ensure that the detoxification part of the vaccine manufacture process is complete. The current pertussis toxin detection test involves immunization with a pertussis toxin-containing vaccine to induce a sensitized state before challenge with histamine. An *in vivo* Chinese hamster ovary test is proposed to replace the mouse sensitization test used to detect the presence of pertussis toxin in acellular pertussis vaccines, but the consistency of correlation between the *in vivo* and *in vitro* tests is still under discussion (Kataoka et al. 2002).

The animals most commonly used in quality control testing protocols are guinea pigs, mice, rats, and nonhuman primates. Generally, an i.p. or s.c. injection is used, both because an appropriate response can be obtained and because a large number of repeatable inoculations can generally be performed.

Many current vaccines are combinations of antigens. Tests that have been established for each of the individual valences are applied to these vaccines. It is important to ensure that no components of the multivalent vaccine interfere with the testing. The intensity of the antibody response to each individual antigen may vary between mouse strains. For a single vaccine valence, inbred strains produce the most repeatable result whereas an outbred stock that responds sufficiently to all valences may be ideal for measuring the response to a multivalent vaccine. Several inbred strains could also be used to obtain the maximum response to each valence. Work is also ongoing to assess the feasibility of reducing the number of animals required by using the *in vitro* assessment of the immunization response to enable investigators to use the same animal for several components of the multivalent vaccine (e.g., diphtheria, tetanus, and polio in guinea pigs). Immunization of combination vaccines in a single animal to detect the response to multiple antigens has also been used to study potential immunological interferences (Sesardic al. 1999).

For some of the newer vaccines intended to develop a Th1 rather than an antibody response, *in vitro* tests are being developed to assess the effectiveness of the immunization. One example is the establishment of a cytokine profile after antigen stimulation of cells *in vitro* (Metz et al. 2002).

Immunization inducing CMI is also used to test products such as the purified protein derivative used in tuberculosis testing. Animals are immunized with a standard s.c. injection with a sensitizing agent (BCG) followed by i.d. injection of test samples. The diameter of the reaction induced by the delayed-type hypersensitivity (DTH¹) response is measured around the injection site. DTH is an *in vivo* assay of cell-mediated immune function that directly reflects the functions of Th1 lymphocytes.

Inducing Immune Suppression Through Regulatory Cell Modulation

Regulatory T cells represent 5 to 10% of the peripheral CD-4 T cell population. They inhibit immune responses that are potentially harmful and help to regulate pathogen-specific responses by inhibiting or enhancing Th1 or Th2 responses during infections. Modulation of these cells has the potential to treat conditions in which an immune response has an adverse effect (e.g., autoimmune diseases, allergies, and tissue graft rejection) or treat infections in which a "latent state" has developed from successful inhibition of the host response. The ability to inhibit or induce specific regulatory T cell activity appropriately likely includes a combination of appropriate antigens, adjuvants, and costimulatory molecules (Burdin 2005; McGuirk and Mills 2002).

Immunotoxicology

Immunotoxicity is a field of expanding interest. Included in this field are studies using immunization techniques to assess the potential immunogenicity of biological drug products and chemicals. Standardized methods for assessing potential effects of chemicals on animal immune responses have been developed. One example is a rat model using a T cell-dependent antigen (keyhole limpet hemocyanin [KLH¹]) inoculated i.d. or i.v. to assess the immunosuppressive potential of coinoculated chemicals (Gore et al. 2004). Protocols have been developed in the rat that demonstrate the effect of age, gender, strain, and site of antigen inoculation in immunogenicity protocols, using lead as an immunotoxin and KLH response as a read-out (Bunn et al. 2001).

Another example comprises adjuvant arthritis models, which are useful for evaluating potential side effects of immunostimulation, including

new adjuvants. The usual intent of immunization is to create a very specific response to a specific antigen. Nonspecific responses can be useful because they may enhance the level of total response to a given antigen. However, when the nonspecific response is too great, it can initiate or exacerbate adverse interactions of the body's immune system against itself, manifesting as conditions such as rheumatoid arthritis. Exacerbation of adjuvant-induced arthritis models can measure whether there is sufficient enhancement of the nonspecific response to induce an adverse condition (Chiocchia et al. 1993).

Consideration of the potential immunotoxicity of new biologics and drugs is now required prior to regulatory approval. The US Center for Drug Evaluation and Research has made available a guidance document on this subject (CDER 2002).

Fertility Control

Immunization as a means of birth control is of interest for human and certain animal population management. A considerable amount of recent activity has been devoted to examining the impact of immunization against sperm, egg, or hormonal antigens (Delves et al. 2002). Immunization against sperm antigen (Diekman and Herr 1997) or testis/epididymis-specific protein (Orand et al. 2004) has proved to be a successful technique to prevent conception in numerous animal models (e.g., mice, rats, guinea-pigs, and nonhuman primates). Immunization against zona pellucida glycoproteins has been investigated in marmosets (Aitken et al. 1996). Although the results have been mixed, this research was useful for demonstrating the impact, both positive (long-term infertility) and negative (premature decline in primordial follicles), of immunization. Success of oral immunization against rabies in wildlife has also opened the possibility of similar protocols for wildlife antifertility immunization (Stohr and Meslin 1997).

Treatment of Addictions

Preclinical research using immunization techniques in animal models has demonstrated sufficient progress and has led to ongoing human clinical trials for both nicotine and cocaine addiction. The objective of these studies is to generate specific antibodies that will bind with the drug and prevent its entry into the brain. The protocols generally involve immunization with a nicotine or cocaine antigen, hapten-conjugated peptides, or larger immunogenic molecules such as KLH or cholera toxin (Carrera et al. 2001; Cerny et al. 2002; Sanderson et al. 2003). Other drugs for which similar immunization approaches are being investigated include phencyclidine, methamphetamine, and heroin (Kantak 2003).

Immunizing Agents

DNA Immunization

DNA immunization involves the direct introduction of plasmid DNA encoding an antigenic protein, which is then expressed within cells of the organism. Immunization with plasmid DNA-encoding antigenic proteins elicits both antibody and cell-mediated immune responses. The introduction of DNA can be accomplished by simple i.m. or i.d. injections, as well as by propelling DNA-coated gold particles into various tissues, preferentially the epidermis (Partidos 2003). DNA vaccination is applicable to a variety of pathogens and is a useful method for enhancing immune responses. Most of the work on DNA vaccines has been conducted in mice. These vaccines have been demonstrated to protect mice from developing against tuberculosis, severe acute respiratory syndrome, and smallpox. The vaccines have the potential to induce prolonged antigenic stimulation, and because plasmids contain many sections of oligonucleotide with a high concentration of cytosine-guanine dinucleotides (CpG¹ motifs), which are more prevalent in prokaryotic cells motifs, they may also have an inherent adjuvant effect. A summary of recent viral disease animal models for DNA vaccines is available in the literature (Davis and McCluskie 1999).

Protection against viral pathogens is improved when DNA plasmid inoculation is followed by a booster of the same encoded antigen expressed in recombinant viral vectors (Hanke and McMichael 1999; Ramsay et al. 1997). The premise is that boosting avoids a cytotoxic T lymphocyte (CTL¹) response that is too narrow. The results have been promising even in nonhuman primate immunization studies for diseases such as HTLV-1 (Kazani et al. 2001) and HIV (Puaux et al. 2004). The immune responses in BALB/c mice using DNA vaccines encoding protein for *Mycobacterium tuberculosis* in a prime boost protocol were recently demonstrated to be of a type important for protection in tuberculosis (Wang et al. 2004). The results have not translated into successful human clinical trials to date. DNA immunization has also been successfully used to produce MAbs of high specificity and avidity (Tearina Chu et al. 2001).

Small Peptides

The ability to sequence and synthesize peptides has led to efforts to create well-defined vaccines that focus on specific antigenic sites. Isolating and cloning the genes that code for protective immunogens has been successful for producing recombinant peptides (Griffin 2002). T cell receptors (TCRs¹) recognize specific peptides or amino acid sequences in association with class I or II MHC receptors. The intent of peptide-based immunization is to induce a specific response to a range of predetermined microbial antigens that will induce a protective immune response. The potential advantage is that antigens that could interfere with the immune response are avoided and those that enhance the response are selected. Small peptides are often conjugated to larger proteins to enhance their immunogenicity. An effective response usually results from a combination of factors including a balance of B cell and CTL induction. Achieving the appropriate mix of peptides and

adjuvants is understandably challenging.

Chimerics

Vaccine constructs in which DNA encoding for particular viral antigens is inserted into the genome of live or attenuated viruses or bacteria have already been successfully used in commercial animal vaccines and also show promise for human immunization applications (Griffin 2002). Vaccinia virus, avian pox virus, herpes virus, adenovirus, polio virus, BCG, *Salmonella* spp., and *Escherichia coli* are among the vectors used in immunization studies.

Various prime-boost combinations of different recombinant vectors, a single recombinant vector followed by subunit proteins, or a single recombinant vector followed by an inactivated cell-based vaccine are being used to augment protection. One trial using canarypox virus-based feline immunodeficiency virus (ALVAC- FIV¹) boosted with an inactivated feline immunodeficiency virus (FIV¹) cell-based vaccine protected against heterologous FIV challenge (Tellier et al. 1998). Similar strategies are being used in nonhuman primates to induce HIV and human T cell lymphotropic virus immunogenicity (Kazanji et al. 2001).

Delivery of Immunizing Antigens

Transcutaneous Immunization

Topical or transcutaneous immunization is of interest as a method for antigen administration because it is safe, convenient, and requires less antigen and adjuvant. In transcutaneous immunization, antigens are often coadministered with cholera toxin or mutants of heat-labile enterotoxin (LT¹) of *E. coli* as adjuvants, and are applied directly to the skin. This method of inoculation has been demonstrated to provide protective immunity in rats and mice (Berry et al. 2004; Mawas et al. 2004; Tierney et al. 2003). Mouse models have been used preclinically to demonstrate the effectiveness of patches impregnated with LT to induce or enhance the immune response to the toxin itself or as an adjuvant for other vaccines. Additionally, laboratory animal studies have demonstrated that this route of administration with appropriate adjuvants may induce both mucosal antibody and CTL responses that can be important in limiting mucosal infections. Primed, skin-based dendritic cells have been shown to migrate to local lymph nodes and gut mucosa. Mouse studies using cholera toxin have demonstrated antibodies and antibody-secreting cells in the salivary gland and female reproductive tract mucosa after transcutaneous immunization (Belyakov et al. 2004; Gockel et al. 2000; Gupta et al. 2004).

The epidermis contains large numbers of antigen-presenting cells called Langerhans cells, which lie just below the stratum corneum. Because there are differences in the cellular composition of the skin between species, extrapolation of the immune response resulting from this route needs to be done with a more complete understanding of the anatomical and immune system differences. Transcutaneous immunization has been shown to induce systemic responses in mice, cats, dogs, sheep, and humans (Chen et al. 2002).

Intradermal Route

The i.d. vaccination route has been studied in animals and humans. These studies have often researched the use of smaller volumes of antigens that have been previously demonstrated to provide an adequate response on i.m. injection. The reason for testing this route is related to dose volume reduction. The responses to date comparing the immune response between, for example, i.m. and i.d. inoculation of hepatitis A vaccine (Brindle et al. 1994) or hepatitis B vaccine (Gomber et al. 2004) have demonstrated lower antibody levels with an equivalent number of i.d. inoculations of lower volumes, although recent results with flu vaccine have been more optimistic (Kenney et al. 2004).

DNA immunization protocols often target the skin either by direct i.d. injection of the antigen or by coating onto microscopic gold particles and bombarding the skin using a gene gun (Johnston and Tang 1994). It is believed that presentation of inoculated antigen by Langerhans and dermal dendritic cells induces antigen-specific T lymphocyte responses. The antigens are acquired through direct transfection or through uptake of transfected keratinocytes expressing the antigen (Tuting et al. 1998).

Mucosal Route

The mucosal immune response, which is different from the systemic response, is often initiated during the early stages of host invasion and infection. The class and distribution of antibodies are different from those for systemic responses and include high levels of secretory IgAs. Mucosal immunization generally induces local humoral and/or cell-mediated immune responses. These responses occur within the mucosa-associated lymphoid tissue, and because the mucosal immune system is considered to be compartmentalized, the immunization strategy involves directly applying adjuvanted immunogen to mucosal surfaces. There are successful exceptions to this strategy. Mucosal vaccines, both nasal and vaginal, have been studied in rodents and humans as a means of inducing local immunity against sexually transmitted diseases such as HIV or *Chlamydia trachomatis* infection (Ericksson and Holmgren 2002). Stimulation of respiratory immunity by targeting gut mucosa has been demonstrated using oral vaccination protocols in animals against *M. tuberculosis* and Venezuelan equine encephalitis (Doherty et al. 2002).

Protective immunity against extracellular pathogens (e.g., *Vibrio cholerae*) is generally antibody mediated, and protective immunity against intracellular or facultative intracellular pathogens (e.g., *Chlamydia* sp. or *Salmonella typhi*) requires both antibody and CMI responses. Mucosal immunization appears to be promising in both of these cases, but it is necessary for the protocols, adjuvants, and tests of efficacy to incorporate these differences. There has been recent interest in adjuvants to direct Th1 and Th2 responses specifically during mucosal immunization and into delivery systems such as the use of live, attenuated bacterial vectors, viral vectors, or virus-like particles (or pseudoviruses), which act as both carrier and adjuvant (Holmgren et al. 2003).

Oral immunization targets gastrointestinal mucosa and is being used to induce gut, systemic, and lung immunity. Several attenuated live vaccines are currently licensed and include oral polio, cholera, and typhoid vaccines. Oral immunization has been studied in animals for many years as an alternative route of administration for immunization (e.g., diphtheria toxoid in rabbits [Peri and Rothberg 1981]) and in rabies immunization in foxes. A rabbit model has been used to test intranasal delivery of flu vaccine adjuvanted with mutant heat-labile toxin from *E. coli* (Pink and Kieny 2004).

DNA vaccination is often directed at the mucosa. DNA administered intranasally in mice has shown rapid and even systemic distribution of the plasmid; however, the DNA disappears rapidly from the lymph nodes and may accumulate in the brain (Ericksson and Holmgren 2002). Topical administration of DNA plasmids with immunostimulatory oligonucleotides as adjuvants is being studied as an immunization technique. Intranasal, intravaginal, and oral administration of CpG DNA has been shown to be effective not only for induction of both mucosal and systemic antigen-specific immune responses against purified protein antigens in animal models, but also in a genital herpes mouse model (Harandi and Holmgren 2004).

Just as direct inoculation of the target mucosal surfaces is important for inducing protective immune responses against pathogens, it is equally relevant for immune responses to induce specific sensitization. It has been reported that i.p. sensitization of BALB/c mice with a fungal sensitization agent did not induce the same level of respiratory responsiveness as an intratracheal route (Ward et al. 2000).

A combined protocol of parenteral and mucosal immunization is used effectively to enhance IgG and IgA mucosal antibodies in nonhuman primates (Israel et al. 1999). Combinations of oral and transcutaneous immunizations have also been studied in mice to induce both mucosal and systemic humoral responses (John et al. 2002).

Lymph Node and Spleen Routes

The inoculation of small peptides directly into lymph nodes has been performed to enhance immune response to small peptides and poorly immunogenic molecules. The concentration of immune cells within lymph nodes enhances the probability of antibody production by increasing the amount of antigen exposed to circulating lymphocytes. Direct inoculation can be performed using a minor surgical procedure, or transcutaneously into superficial lymph nodes such as the popliteal lymph nodes.

Early evidence suggested that direct lymph node inoculation generally provides the greatest systemic response against poor immunogens, followed by intra-articular, i.d., i.m., i.p., s.c., and i.v. routes (Mitchison 1972). Antibodies to nanogram quantities of immunogen have been achieved in vitro by presenting the immunogen to a spleen cell culture, and in vivo by introduction of the antigen through intrasplenic immunization (Nilsson and Larsson 1990).

Intraperitoneal and Intramuscular Routes

The i.p. immunization route for administering an immunizing antigen is convenient and capable of inducing systemic responses. However, it is often less physiologically relevant than other routes, as demonstrated in immunization protocols to induce sensitization against fungal antigens in mice (Ward et al. 2000). Inoculation by the i.m. route also is convenient, and this latter route compartmentalizes the antigen and adjuvant to facilitate processing by macrophages, providing for some consistency of systemic responses.

Adjuvants and Delivery Systems

A wide variety of adjuvants are used in immunization protocols, and selection is based largely on the desired response. Considerations for using specific adjuvants involve a balance between trying to attain a high degree of directed immune responsiveness and minimizing adverse nonspecific inflammatory consequences. Sequelae can include local reactions or granulomatous pneumonia and other systemic responses. The only adjuvants currently used in licensed human vaccines are aluminum based. A number of novel adjuvants are already being tested, or are close to testing in human clinical trials, including IL-12 cytokines and antigen-loaded dendritic cells (Pink and Kieny 2004). Many of these delivery systems and adjuvants are being developed to target mucosal immunity and to enhance Th1 responses (Moingeon et al. 2002). The s.c. immunization of rabbits with nitrocellulose paper strips impregnated with small amounts of antigen has been performed without additional adjuvant (Coghlan and Hanausek 1990).

Traditional whole cell vaccines have contained immune potentiators in addition to protective antigens that have stimulated a robust response. Newer subunit vaccines based on purified recombinant proteins, synthetic peptides, and plasmid DNA tend to have a poorer antigenic response, and effective adjuvants become more important. Lipopolysaccharides, lipopeptides, and CpG motifs have been used as

immunomodulators. In addition, formulations and carrier systems such as emulsions, liposome microspheres, and immunostimulant complexes are used to increase the immunogenic response (Lima et al. 2004). In an attempt to induce T cell responses through the phagocytic pathway, particulate vaccines have been created from antigens bound to beads. These vaccines have been assessed in murine models and in ponies challenged with the equine infectious anemia lentivirus (Hammond et al. 1999).

It is beyond the scope of this article to review adjuvant properties. Therefore, we refer the reader to several recent reviews (Alving 2002; Moingeon et al. 2002; O'Hagan and Valiante 2003; Pink and Kieny 2004; <http://www.niaid.nih.gov/daids/vaccine/pdf/compendium.pdf>).

Animal Immunization Protocol Considerations

The immune response is a complex system of physical barriers, physiology, antigen processing and presenting cells, cytokines, and effector cells. The choice of immunization protocols is equally complex because many considerations can influence the overall result, such as the dose and concentration of antigen, the choice of adjuvants, the time between inoculation and response measurement, and the method of detection.

To understand the responses generated to an immunization protocol, it is important to understand the features of the model being used. The selection of an appropriate animal model is a key consideration. Questions that must be considered during these deliberations include the following:

- What is the relevance of the species to the question being investigated?
- What is the specificity of the result that will be detected from that species?
- What other genetic, hormonal, or environmental influences may affect the result?

Similarly challenging is the determination of an appropriate inoculation schedule. Careful consideration is necessary in selecting the following:

- Dose and schedule;
- Optimal concentration, volume, and dose range of the inoculum
- Purity of the antigen; and
- Types of product and adjuvant preparations required.

For booster inoculations, the following aspects require careful consideration:

- Frequency;
- Whether they must be administered in the same conditions or a different form, adjuvant, or site; and last but importantly, the
- Purpose of the study, which can influence the dose, animal species, strain, and number of animals (e.g., depending on whether for quality control testing, increasing hyperimmune sera, or research and development).

Inoculation Dose and Procedure

The optimal inoculation dose varies for different antigens. Sufficient antigen is required to induce a response vigorous enough to activate the immune cascade fully. Higher doses can generate larger amounts of antibody; however, they may be of lower affinity, and even higher doses tend to induce immunological tolerance. Dose selection often requires pilot dose response studies with a wide range of antigen dilutions. Pilot study criteria may be refined by basing it on existing information about similar antigens. A useful review of protocols for antibody production (Hanly et al. 1995) includes guidelines for injection quantities, sites, and schedules. Volumes that are administered are relative to the size of the animal. As discussed, the site is important for compartmentalization of the response and the response sensitivity. Inoculations using the same route but in different anatomical sites can in some cases influence the intensity of immune responses, as has been shown with gluteal i.m. responses compared with deltoid i.m. responses for hepatitis B vaccine (Shaw 1989). Injection technique is also important. For example, a large percentage of i.p. inoculations may not end up i.p. (Miner et al. 1969).

Booster

Boosters can be repetitions of the original inoculation, or they can vary in dose, adjuvant, vector, and site of inoculation. The combined objective of booster inoculations is to take advantage of natural immune responses to infections and to induce a stronger, longer lasting, and more specific immune response. This response is quantified by higher antibody concentrations and persistence of the antibodies. The secondary immune response induced by boosting activates memory cells that are formed after the primary inoculation and has the following characteristics: a shorter latency phase before antibody production; a faster log phase of increasing antibody concentration; more IgG, E, or A and less IgM; and antibodies of greater affinity and avidity.

The optimal schedule for maximizing antibody production varies with species, antigen, and adjuvant. Generally, optimal results occur using booster doses at least 4 wk after the first injection. Antibody levels increase to a maximum approximately 10 days after each booster in

guinea pigs and rabbits. Published reports in rabbits have shown a peak antibody production 12 days after the last dose of a multiple inoculation protocol and a return to basal levels after 60 days. Administration of antigen in the presence of existing antibody does not produce high-titer antibody (Chande et al. 1996). Increasing the frequency of booster doses does not necessarily enhance the concentration and avidity of antibody production, and has been shown in some cases to have the opposite effect. The impact of boosters can depend on the concentration of antigen used (Hu and Kitagawa 1990; Hu et al. 1990). A long rest period before boosting can enhance the avidity of the response. These studies also indicate that too high a concentration can induce tolerance, and too frequent boosting can diminish antibody response and avidity. The same phenomenon of too frequent inoculations resulting in diminishing response is seen with CTL stimulation (Serody et al. 2000).

Detection/Measurement of Immune Response

In the absence of a challenge or disease model, classic immune responses have been detected using antibody quantification. Even with antibody detection, it is important that the antibodies measured are relevant and protective, and that the method has specificity. With expansion of immunization objectives, there must be standardization of monitoring, quantification, and comparison of efficacy of other responses (e.g., T cell-mediated responses for cancer and intracellular infectious agent studies). This is an area of active investigation, and many methods have been developed, such as immunospot assays and cytokine flow cytometry to measure developed cytokines, and soluble recombinant MHC-peptide tetramers to measure levels of T cells, with antigen-specific TCRs (Keilholz et al. 2002; Lysterly 2003). There continues to be use of DTH to measure CMI responses in animal models (Augustinova et al. 2004). The DTH response can be analyzed quantitatively and qualitatively (Nichols et al. 2002).

Animal Model Selection

Species

Although there is conservation of many immune system mechanisms and cell types between mammalian species, there is also great variability in responses to specific antigens and adjuvants. An understanding of the similarities and differences between species is essential for appropriate selection of animal models and data interpretation. DNA immunization alone has been successful in mice but has not had the same direct success in dogs, nonhuman primates, or humans. Studies using the same adjuvant--MF59, an oil/water emulsion containing squalene--have shown large differences in the intensity of responses between species. The ratios of titers obtained from inoculation of antigen using MF59 compared with the same antigen and alum were twice as high in baboons compared with guinea pigs for hepatitis B virus surface antigens; yet for herpes simplex virus gD2 antigen, the guinea pigs had a seven times higher response (Alving 2002).

The guinea pig model used in diphtheria vaccine potency testing, as an example, has many characteristics of a preferred animal model. It is susceptible to the diphtheria toxin, and has been used in protection studies. It produces antibodies to the relevant diphtheria toxin B-fragment domain, whereas mice do not produce antibodies at similar dose levels. Similar serum antibody dose responses to the diphtheria toxoid are seen, irrespective of the detection method used, and there is a serological response to functional epitopes. Guinea pigs are available from commercial suppliers, and adequate amounts of sera can be recovered for analysis.

Mice are frequently used in immunization studies to test for proof of concept and for understanding of key immune response data. Avian species are also used. The murine immune system, including the roles and interactions of different cellular components, has been well defined and is similar enough to the human system to permit cross-species comparisons. The use of humanized mice (i.e., SCID mice reconstituted with human immune cells [Mosier et al. 1993]) and transgenic mice can provide rapid answers to specific human immune cell responses in an in vivo model. Transgenic mouse lines that express human MHC class I and class II genes, with or without a mouse H-2 class I knockout, may be used in immunization protocols to analyze the potential human CTL responses to specific antigens. In this regard, these mice may be used to screen potential vaccine antigens and to test vaccination strategies (Firat et al. 1999; Palmowski et al. 2002). For the study of immune responses to intracellular pathogens, where there are complex interactions between the pathogen and the host immune system, the extrapolation of protective immunity from mice and rats directly to target species may be limited when they are not the naturally infected hosts. The pathogenesis of infection in an animal model should mimic that found naturally, and the patterns of immune response should also be similar to the naturally infected host (Griffin 2002).

Animal models can be used to demonstrate the effectiveness of immunization against specific disease conditions (Griffin 2002). Both direct infectious agent challenge and control of natural disease in a population are used to demonstrate disease prevention, and pre-existing or induced disease models are used for therapeutic immunization. Agencies that regulate vaccines recommend that when possible, new vaccines and adjuvants should be tested for safety in a species in which a postvaccination challenge can be studied (Pink and Kieny 2004). There may be a range of animal models susceptible to a specific disease, but the model selected should depend on the objectives of the study. Herpes viruses, for example, infect a range of animal species. Guinea pigs and nonhuman primates have been used for immunogenicity studies, mice and primates for protection studies, rabbits for ocular infection and mice, and guinea pigs and rabbits for latent infection (Methner 2001).

Although nonhuman primates often predict human responses, the value of this candidate should also be evaluated in human clinical trials. Even then, among humans, there is tremendous regional genetic variability, and extrapolations about consistency of immune responses between regions must be made with caution. For example, West African populations have increased frequencies of an HLA-B*5301 allele that controls the binding of a liver stage-specific antigen of *Plasmodium falciparum*, which confers a high degree of resistance to malarial

disease (Hill et al. 1992).

Animal size also influences animal model selection, particularly when the objective of immunization is to manufacture immunoglobulins on a large scale. This goal is traditionally accomplished in large animals such as horses. Advances in diagnostic techniques for antibodies and immune cells are becoming more sensitive. In other words, smaller sample volumes can provide the same information. New technologies such as multiplex microsphere immunoassays allow quantification of antibodies to several different antigens using the same serum sample. As mentioned above, the ability to detect multiple antigens from the immunization of a single animal also has the potential to reduce the number of animals used in quality control testing of vaccines.

For alternate delivery systems and new sites of immunization, anatomical and immune system differences between these mice are being considered. The lung, uterine, placental, and skin anatomy as well as the immune cell architecture vary greatly between species, and this variation has obvious implications for extrapolation and comparison of data, especially for the study of immune responses to intracellular pathogens that have complex interactions between the pathogen and the host immune system.

Inbred and Outbred Strains

Vaccine testing provides examples of the requirements for inbred and outbred animals. If the intent is to use the model to simulate the robustness of results on a highly varied population such as the general human population, then an outbred animal may be more appropriate. If the objective is to demonstrate consistency or repeatability of a response, such as ensuring consistent vaccine immunogenicity, then an inbred strain, with the control of genetic variation that this strain implies, enables the comparison. Strain differences in intensity of immune responses to specific antigens in mice and rats have been well documented for both antibody and cell-mediated immunity. Differences in response to immunization vary greatly among inbred mouse strains (Melo et al. 2002) and between pig populations (Wilkie and Mallard 1999) and are likely antigen dependent. This phenomenon exists in outbred populations but is less obvious. For example, CD rats produce higher antibody responses, whereas F344 rats exhibit elevated DTH responses to KLH immunization.

Thus, the choice of species, strain, or stock of animal may have a significant influence on the intensity of the response observed. Even for a single strain of animal, responses vary between suppliers. A recent study examined immune responses to vaccine antigens in mice and guinea pigs from different breeders by comparing i.m. immunizations with HIV-1 antigens presented by a canarypoxvirus vector (the ALVAC-HIV [vCP205] virus) and with recombinant proteins (e.g., detoxified Tat_{IIIB} or adjuvanted gp160 MN/LAI-2 in guinea-pigs, and intranasal adjuvanted gp160 MN/LAI-2 in Swiss, OF1, CD1, NMRI, BALB/c, and C57 BL/6 mice). Even if all of the animals responded to the antigens tested, variations in the intensity of the immune responses were observed. In this regard, the origin of the mice as well as the strain used should be taken into account when comparing results obtained between laboratories (Jourdiere et al. 2005).

Age and Flora

The immune system is a state of equilibrium that changes as the organism and the components of the immune system mature (Wakabayashi et al. 1999). When the animal is an infant, initial antibody responses are primarily IgM based and nonspecific. As the animal matures, the responses become more IgG/A/E based, with an improved memory response. The maturity of the immune system is influenced by previous immune challenges. There is a constant challenge of the immune defenses by organisms crossing the physical barriers of skin and mucosa. These challenges have a positive effect, enhancing the rapidity of the immune system response if the host encounters similar antigens later in life. Challenges can have a negative influence when antigenic tolerance develops, or when immunosuppression results from a latent, concurrent infection.

Susceptibility to infection and immune system response also change over time (Gutowski and Weksler 1982; Sheridan et al. 1983). The extent to which the immune response varies with time is dependent on species, strain, and sex (Hosono et al. 1986; Krzych et al. 1979; Walters and Claman 1975). Even within the same strain, the maturation of the immune response can be influenced by environmental factors such as commensal organisms. Intestinal microflora directly influences the regulation of peripheral T cells (Klaasen et al. 1993; Pickard et al. 2004). This effect is important when selecting the age and health status of animal models for studies and when comparing results generated even from the same species and strains.

The age of the animal is considered to have a greater impact on the immune response than its weight, yet weight is still considered a defining parameter in some vaccine protocols. The prevailing importance assigned to weight is possibly due to a general association between animal weight and maturity within particular species. This association can be influenced significantly by diet, environment, and genetic drift. A study examining the optimal immunization schedule in mice using viomycin as a model immunogen demonstrated that age was more important than sex, strongly affecting both total and specific IgG levels. In this study 8-wk-old mice and yielded the highest levels of antibody (Hu et al. 1990).

It is the combination of normal age-related development and immune response to external challenges that evolves into the immune response against a specific antigen (Van Oudenaren et al. 1984). Specific pathogen-free (SPF¹) animals can have immune responses that differ from conventional animals and from other SPF animals exposed to different commensal flora (O'Rourke et al. 1988). The normal flora can influence both gene expression in antigen-presenting cells and the way T cells respond to vaccines (Klaasen et al. 1993).

Sex and Hormonal Status

Sex and hormonal differences are also factors to be taken into account in immune system responsiveness. In one guinea pig study, the antitoxin titers to tetanus toxoid after a single inoculation were consistently lower in females than in males. This difference was not observed after a booster immunization (Rethy et al. 2001). These findings suggest that levels of circulating hormones can have a marked effect on the immune system.

The stage of the estrus cycle at the time of immunization may also influence the immune response. This potential effect has been demonstrated in a study using female BALB/c mice in which oral immunization with tetanus toxoid and cholera toxin enhanced tetanus toxoid response in the female reproductive tract during estrus. Not all routes of immunization were affected similarly. Tetanus toxoid-specific T cell proliferation was greatest following intranasal administration at proestrus and after transcutaneous administration at diestrus (Gockel et al. 2003).

Stress in animals affects the immune response. Shipping cattle or mixing unfamiliar pigs has been shown to suppress immune responses to vaccination (de Groot et al. 2001). This is one of the main reasons that an appropriate acclimation period is required after periods of stressful events such as transportation, before experimental procedures are initiated.

Nutrition

Nutritional status, particularly nutritional insufficiency, is also known to affect immune responses. The importance of this factor has been demonstrated in guinea pigs provided isocaloric diets containing 30%, 20%, or 10% casein and vaccinated with viable BCG. Skin test responses to purified protein derivative, a measure of DTH response, were absent or suppressed in the 20% and 10% casein-supplemented groups along with other indicators of CMI stimulation (McMurray and Yetley 1982). Protein deficiency has been demonstrated to affect thymic function and immunological maturation in rats (Konno et al. 1993). Supplementation with vitamins A, E, and C and with selenium has been demonstrated to enhance immune response in specific circumstances such as heat stress, weaning, or increased growth periods (Lin et al. 2002; Swain et al. 2000; Zhao et al. 2002).

Concluding Remarks

The process of immunization has had a tremendous impact on the lives of humans and animals in providing protection from many infectious diseases through vaccination. In addition, the increased understanding of immune systems has led to new applications of immunization procedures. Scientists are at the cusp of developing an astonishing array of novel and promising therapies for other infectious diseases as well as cancer, allergies, addiction, and autoimmune and degenerative diseases. It is indeed noteworthy that *all* of the forementioned sources of protection and treatment have been developed using knowledge gained from immunization protocols.

The immune system responses to an antigen are complex and continue to develop with individuals' advancing age. Anatomical, physiological, and immune system differences between species influence responses to immunization, as do the purity and presentation of the antigens and adjuvants. When directly comparing results, animals should be sourced from the same supplier.

Published immunization protocols are sometimes extrapolated for use in different institutions. It is imperative that such extrapolations are made with care and with an understanding of why the model was selected. A review of models and protocols, combined with preliminary studies, can help ensure an appropriate selection of immunological procedures. This thorough approach will avoid the generation of redundant data while ensuring continued benefits from new forms of immunological therapies.

¹Abbreviations used in this article : ALVAC-FIV, canarypox virus-based feline immunodeficiency virus; BCG, Bacille Calmette-Guérin; CMI, cell-mediated immunity; CpG motifs, sections of oligonucleotide with a high concentration of cytosine-guanine dinucleotides, more prevalent in prokaryotic cells; CTL, cytotoxic T lymphocyte; DTH, delayed-type hypersensitivity; ELISA, enzyme-linked immunosorbent assay; FIV, feline immunodeficiency virus; HIV, human immunodeficiency virus; HLA, human lymphocyte antigen; Ig, immunoglobulin; IL, interleukin; KLH, keyhole limpet hemocyanin; LT, heat-labile enterotoxin; MAAb, monoclonal antibody; MHC, major histocompatibility complex; SCID, severe combined immunodeficient; SPF, specific pathogen-free; TCR, T cell receptor; TLR, toll-like receptor; TNF, tumor necrosis factor; YAC, yeast artificial chromosome.

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