Vaccines, Adjuvants, and Delivery Systems for Infectious Diseases

Suresh K. Mittal
Harm HogenEsch
Ramesh Vemulapalli
Department of Comparative Pathobiology, Purdue University, West Lafayette, Indiana, U.S.A.

Kinam Park
Departments of Pharmaceutics and Biomedical Engineering, Purdue University, West Lafayette, Indiana, U.S.A.

INTRODUCTION

The best approach for reducing the death and suffering of humans and animals due to viral, bacterial, and parasitic diseases is prevention using effective vaccines. The first scientific basis for vaccination was introduced by Edward Jenner in 1796 when he used cowpox virus as a vaccine to protect humans against smallpox virus infections. This was the first evidence of successful use of a naturally available attenuated virus as a vaccine, and it happened without the knowledge of the concept of viruses or even infectious pathogens. Over the past two centuries, especially after 1940, a number of vaccines have been developed to provide protection against infectious diseases. With a few exceptions, the technology of vaccine development and production has not changed significantly over the years. Vaccination usually involves the use of either a killed pathogen combined with an adjuvant or a live pathogen with reduced virulence. These two types of vaccines are effective only against some pathogens, and a few of these vaccines have some undesirable side effects. Additionally, this traditional approach for developing an effective vaccine has been unsuccessful against a number of important pathogens. Other several potential vaccine candidates have been identified as protective viral (envelope and/or capsid proteins or glycoproteins and other viral proteins), bacterial (surface, internal, or fimbria proteins; bacterial polysaccharides; bacterial toxins; and other proteins involved in bacterial metabolism), or parasitic (various stages surface or internal proteins) antigens. The potential of these protective antigens are being explored in order to develop effective vaccines. In addition to prevention of infectious diseases, the field of vaccine technology has shown potential in other areas, such as cancer treatment, reproduction, and modulation of animal productivity. An overview of vaccine strategies is depicted in Fig. 1.

VACCINE STRATEGIES

Live Attenuated Vaccines

A live attenuated vaccine constitutes the use of a live agent as a vaccine. A disease causing pathogen can be attenuated in such a way that it loses pathogenicity for its host but retains immunogenicity. For some pathogens, even a virulent agent can be used if a different route of inoculation is used to circumvent infection leading to the disease. For example, human adenovirus serotypes 4 and 7 are involved in acute respiratory infections in humans when administered via the oronasal route, but provide protection when given orally in enteric-coated capsules (1).

The method of attenuation is largely dependent on the infectious agent against which an effective attenuated vaccine is needed. Some organisms can be attenuated by growing them under abnormal conditions which include cultivation in unnatural hosts or cell lines or replication at different temperatures, nutrients, and/or pH levels. In the case of viruses with a segmented genome (e.g., influenza virus, reovirus, birnavirus), attenuated viruses can be obtained by the process of recombination of genome segments in a mixed infection known as genetic reassortment. This process of reassortment has led to the development of a reverse genetics approach to easily change gene segments of a segmented genome virus (2). A wild-type pathogen of one host may be attenuated for another host, e.g., vaccinia virus served as an attenuated virus vaccine for the small pox virus eradication program during the 1960s and 1970s, and turkey herpes virus is still used as an attenuated vaccine for Marek's disease virus (chicken herpes virus). The Bacille Calmette–Guerin (BCG) strain of *Mycobacterium tuberculosis* was attenuated after more than 200 passages in media containing increasing amounts of bile. The Sabin polio virus vaccine was attenuated by a number of passages in monkeys and in monkey kidney epithelial cells (3). Measles virus was initially adapted to monkey kidney cells and subsequently attenuated in duck embryo and human tissue culture cell lines (4–6). Orally administered attenuated *Salmonella* are known to interact with the mucosal-associated lymphoid tissue (MALT) (7). Table 1 describes the nature of attenuated vaccines that are currently being used for human applications.

Temperature-sensitive (ts) mutants have proven to be the most useful type of mutants for a number of viruses and bacteria because of their conditional lethal phenotype. The ts mutants are produced by alteration of the nucleotide sequence of a gene so that the resulting protein product is

Encyclopedia of Pharmaceutical Science and Technology DOI: 10.3109/9781841848204.000: Vaccines, Adjuvants, and Delivery Systems

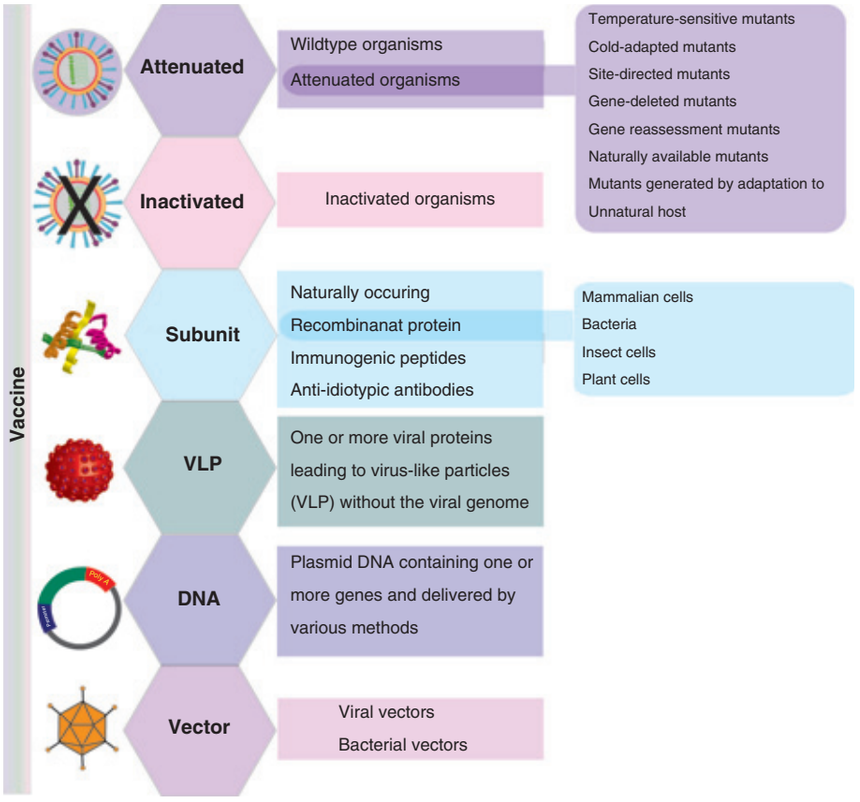

Figure 1 Overview of various vaccine strategies.

unable to assume or maintain its functional configuration at the non-permissive (37–39°C) temperature. The protein, however, is able to assume a functional configuration at the permissive temperature (32–34°C), e.g., herpes virus, adenovirus, and influenza virus. Thus, these mutants can replicate in mucosal sites with a lower temperature, e.g., the nasal cavity, but are unable to cause a systemic infection and clinical disease.

Gene-Deleted Vaccines

Identification of the virulence factor(s) of a pathogen is important in generating an attenuated pathogen that could provide effective protection if used as a vaccine. Some earlier versions of attenuated vaccines contained random mutations in the genomes of their respective pathogens. If random mutations are point mutations, attenuated agents may regain virulence owing to back mutations. Due to increased understanding of the virulence of various pathogens at the molecular level, one or more genes responsible for virulence have been identified for many pathogens. The genes associated with virulence may be genes involved with nucleic acid replication and other non-structural and structural components of the organism. A partial or complete deletion of one or more virulent genes could lead to the development of better attenuated vaccines compared to attenuated agents having point mutation(s).

Swine herpes virus has been attenuated by deleting genes associated with viral virulence. These genes include the thymidine kinase gene (non-structural protein involved in viral DNA replication) and the gC, gG, and gE genes (non-essential glycoproteins involved in virus assembly) (24,25). A gene-deleted vaccine of pseudorabies virus has proven highly effective in eradicating psuedorabies in the United States. It has been demonstrated that *Salmonella typhimurium aroA, aroB*, and *aroC* deletion mutants fail to grow in a host because of the absence of aromatic amino acid production. These genes have been targeted to reduce the virulence of the bacterium. *S. typhimurium* gene-deleted mutants are capable of replication for at least a short period in the host, thus raising a protective immune response (26). Vaccination with gene-deleted vaccines also allows eradication of wild-type pathogens from the population. Because antibodies against the deleted gene product will only be developed in infected animals, it is feasible to differentiate between vaccinated and naturally infected animals (27,28). The process of gene deletion not only attenuates the pathogen, but also offers a unique opportunity to insert foreign genes for developing viral or bacterial-vectored vaccines.

An attenuated agent replicates in its host without causing disease symptoms, thereby leading to induction of an immune response similar to a natural infection with the disease-causing organism. A number of advantages associated with live vaccines include: (i) low cost of production due to fewer numbers of organisms in the vaccine formulation; (ii) fewer number of inoculations; (iii) no need for an adjuvant; (iv) induction of both humoral and cell-mediated immune responses; and (v) use of the natural route for vaccine delivery. Some of the disadvantages associated with live vaccines include: (i) stability and storage requirement; (ii) possibility of reversion to virulent form; (iii) may cause

Table 1 Live attenuated vaccines for use in humans

Target pathogen	Vaccine strain	Attenutation method	Gene/Protein responsible for attenuation	References
Varicella-zoster virus	Oka/Merck	Passage in human and guinea pig embryo fibroblasts	Not defined	(8–10)
Yellow fever virus	17D-204	Passage in duck embryos	E and NS2a	(11,12)
Salmonella typhi	Salmonella typhi Ty21a	Chemical mutagenesis	galE	(13,14)
Rubella virus	Wistar RA 27/3	Passage in WI-38 human diploid lung fibroblasts	Not defined	(15,16)
Rotavirus	human 89–12	Passage in monkey kidney cells, genetic reassortment between human and bovine rotavirus strains	Not defined	(17)
Mumps virus	Jeryl Lynn	Passage in chick eggs and chick embryo fibroblast cells	Not defined	(18)
Measles virus	Enders' attenuated Edmonston	Passage in Vero cells	Not defined	(19)
Influenza virus	Reassortant master donor viruses A/Ann Arbor/6/60 and B/Ann Arbor/1/66) containing HA and NA genes from wild-type influenza A and B viruses	Genetic reassortment between attenuated master donor virus and circulating wild-type viruses	PA, PB1, PB2, and NP	(20–22)
Mycobacterium tuberculosis	rBCG30	Culture in bile containing media	30 kDa protein gene	(23)

disease in immunosuppressed, immature, older, or pregnant hosts; (iv) the agent may be attenuated for all hosts; and (v) possibility of contamination with other adventitious agents. For example, earlier batches of polio vaccines in the early 1960s were contaminated with simian virus 40. The current screening methods for monitoring the absence of adventitious agents in the certified cell lines or vaccine preparations provide enhanced safety of attenuated vaccines.

Inactivated Vaccines

The creation of an inactivated vaccine involves inactivating a pathogenic agent in order to block its replication, thereby allowing the immunogenic antigen(s) to serve as a vaccine when inoculated into a normal immunocompetent host without causing infection. Inactivated vaccines are also known as killed vaccines. This is the simplest way to produce vaccines, provided that the organisms can be cultured easily and that the antigen(s) in the mixture provides effective protection. Therefore, this approach is the most likely starting point for vaccine development against new or emerging pathogens. However, inactivated vaccines are effective against only some infectious agents. An infectious agent can be inactivated by a chemical (e.g., formalin, formaldehyde, or beta-propiolactate), heat, or gamma-irradiation. In some instances, the inactivation procedure may enhance the antigenicity of some antigens important in protection. Typically, an inactivated vaccine elicits high levels of humoral immune response after multiple inoculations. In general, an inactivated vaccine results in low or insignificant levels of mucosal and cell-mediated immune responses; therefore, it may provide only limited protection against a mucosal or intracellular pathogen.

Failure to completely inactivate a pathogenic agent could result in disease instead of protection. During the 1950s, some lots of poliovirus vaccine were not inactivated completely resulting in polio infection in those receiving the vaccine (29,30). Since current methods for detecting residual infectivity are more stringent, inactivated vaccines are considered safe with an extremely low or no chance of infection leading to clinical signs of disease. There have been other instances wherein inactivated vaccines led to an atypical disease or enhanced disease severity. For example, in the 1960s, formalin-inactivated respiratory syncytial virus (RSV) vaccine actually enhanced the disease's severity when immunized children were naturally exposed to RSV (31,32). It was later determined that a change in the antigenicity of RSV F and G glycoproteins (33) resulted not only in alterations to the humoral immune response,

Table 2 Inactivated vaccines for use in humans

Target pathogen	Attenuation methods	Preparation	References
Anthrax	Formalin	Extract of attenuated bacteria	(35)
Polio virus	Heat and formalin	Inactivated virus	(36)
Hepatitis A virus	Formalin	Inactivated virus	(37,38)
Rabies virus	β-Propiolactone	Inactivated virus	(39,40)
Japanese encephalitis virus	Formaldehyde	Inactivated virus	(41,42)
Influenza virus	Formaldehyde	Inactivated virus	(43)
Clostridium tetani	Formaldehyde	Tetatnus toxoid	(44)
Corynebacterium diphtheriae, Clostridium tetani, Bordetella pertussis	Glutaraldehyde and formaldehyde	Diphtheria and tetanus toxoids, acellular pertussis vaccine adsorbed	(45)

but also in the Th1 and Th2 components of the CD4+ T-cell response to RSV (34). The currently available inactivated vaccines for human applications are listed in Table 2.

Subunit Vaccines

A subunit vaccine consists of one or more immunogenic proteins, epitopes, or other components of a pathogenic organism. Immunogenic epitopes can be chemically synthesized and are known as peptide vaccines, e.g., peptide vaccine candidates for foot-and-mouth disease virus (46,47). To produce a subunit vaccine, the pathogen is disrupted, and one or more immunogenic proteins such as bacterial cell wall proteins, flagella or pili, viral envelope, capsid, or nucleoproteins are purified. These purified proteins then form the basis for the subunit vaccine. The isolation of such components in purified form is sometimes cumbersome and expensive. However, bacterial exotoxins can be easily purified, inactivated, and used as toxoid vaccines.

Subunit vaccine production can be done in a variety of expression systems including bacteria, yeasts, insect cells, mammalian cells, and plant cells. The choice of expression system depends on the nature of antigen. High amounts of a foreign protein can be produced in a bacterial expression system at a low cost because the scale-up and downstream processing have been well worked out for bacterial expression systems. A particular bacterial expression system, however, may produce proteins of altered immunogenicity because the bacterial system lacks many post-translational processes. Other immunogenic proteins, especially of viral origin, require secondary modifications that are important for their antigenicity. Some viral glycoproteins expressed in bacteria induce protective immunity, e.g., gp70 of feline leukemia virus (48). A yeast-expressed hepatitis B virus surface antigen–based subunit vaccine is currently in use for humans and has demonstrated excellent protection efficacy (49). This vaccine is an excellent example of the potential of recombinant subunit vaccines for providing protection against many viral and bacterial infections.

Table 3 Subunit and conjugate vaccines for use in humans

Target pathogen	Target antigens	Vaccine type	References
Hepatitis B virus	Hepatitis B surface antigen	VLP	(56,57)
Human papilloma virus	L1 protein from HPV types 6, 11, 16 and 18	VLP	(58,59)
Human papilloma virus	L1 protein HPV types 16 and 18	VLP	(60)
Neisseria meningitidis	Meningococcal polysaccharides	Subunit	(61,62)
Streptococcus pneumoniae	Capsular polysaccharides	Subunit	(63,64)

Mammalian cells are known to process animal virus glycoproteins to their functional form by secondary modifications; hence, they are considered one of the means to produce viral antigens where secondary modifications are a critical part of their antigenicity. Expression levels of protein expressed in mammalian cells are usually lower compared to the bacterial or yeast expression system. Production of subunit vaccines in mammalian cells is usually expensive because of the low level of this foreign gene expression and high processing cost. Stable expression of the transmembrane anchor-deleted form of many viral glycoproteins in mammalian cells results in the secretion of truncated products in the medium in large quantities that can be used as a subunit vaccine without further purification. However, the removal of the transmembrane anchor may potentially alter the antigenicity of the secreted protein. There are a number of viral glycoproteins that are expressed either in mammalian or insect cells and the secreted proteins have been found to be suitable candidates for providing protective immune responses. These include the F and G genes of respiratory syncytial virus (50), the HN and F genes of the parainfluenza virus (51), the gD gene of bovine herpes virus type 1 (52), and the hemagglutinin (HA) gene of influenza viruses (53–55). Information on the currently used subunit vaccines in human are presented in Table 3.

Immunogenic Antigen Production in Plants

Significant progress has been made in the stable integration and expression of a wide variety of genes in plant cells, resulting in the creation of novel plants for agricultural and industrial use. The inserted genes confer resistance to insect pathogen and herbicides; enhanced tolerance to drought, salt, and frost; and improved agricultural production. Undoubtedly, improvements in plant attributes by genetic engineering will have a great impact on agriculture production. However,

it has been estimated that the major economic gain of plant biotechnology will result from the use of plants as bioreactors to produce high-value products such as vaccines, industrial enzymes, and other pharmaceuticals.

Transgenic plants offer an attractive alternative to produce functional viral, bacterial, or parasitic proteins in large quantities at a very low cost for a subunit vaccine compared to other methods. Production of subunit vaccines in mammalian cells is usually expensive because of the low level of foreign gene expression and high processing cost. While high levels of foreign gene expression can be obtained in bacteria and yeast, many animal virus proteins expressed in these systems fail to undergo proper secondary modifications such as glycosylation, phosphorylation, sulfation, etc. Therefore, these recombinant proteins may have altered antigenicity. However, the mechanisms regulating secondary modifications of proteins are already present in plants (80). Similarly, the production of functional multimeric antibody molecules in plants has made it possible to manufacture antibodies in bulk amounts for passive immunization (81).

Two major strategies have been devised to produce foreign proteins in plants. These are: (1) stable integration of a chimeric gene into the plant genome under suitable constitutive or inducible plant promoters, (82,83), and (2) manipulation of plant pathogenic viruses (84). Foreign protein expression in plants usually ranges from 0.01% to 1% of the total plant protein. Injection of hepatitis B virus surface antigen produced in transgenic tobacco in mice elicits a protective immune response (85). Mice fed transgenic potato tuber-expressing B subunit of heat-labile enterotoxin (LT-B) of enterotoxigenic *Escherichia coli* developed antibodies to LT-B, particularly immunoglobulin (Ig)A antibodies. Dalsgaard et al. (82,84) demonstrated that immunization of mink with the VP2 capsid protein of mink enteritis virus, expressed in cowpea after infection with modified cowpea mosaic virus, elicited a protective immune response. Protection occurred against challenge with virulent foot-and-mouth disease virus in mice inoculated with the structural protein VP1 of foot-and-mouth disease virus produced in transgenic Arabidopsis (83). It has been hypothesized that transgenic plants could serve as "edible vaccines", thereby providing a very inexpensive means of oral immunization (86).

Anti-Idiotypic Vaccines

Another approach to provide protective immune response is the use of anti-idiotype antibodies as vaccines. Antibodies have unique sequences in the variable (V) region of their binding site known as "idiotypic determinants". Some of the idiotypic determinants make up the antigen-binding site (paratope) of the antibody. The part of the antibody that binds to the antigen is called a paratope. Antibodies to a specific paratope of an idiotype, which mimic the epitope of an immunizing antigen, are known as anti-idiotypic antibodies. Thus, anti-idiotype antibodies are mirror images of antigens and can be used instead of immunogens to elicit a protective immune response. Monoclonal anti-idiotypic antibodies could serve as a source of antigens. Anti-idiotype vaccines are useful in cases where the actual antigen is poorly immunogenic or similar to host antigens. Some of the pathogens against which anti-idiotype vaccines have been tested include *Listeria monocytogenes*, *Streptococcus pneumoniae*, hepatitis B virus, Semliki forest virus, and Sendai virus (87,88). It is questionable whether this type of vaccine will be available for human use soon.

Virus-Like Particles-Based Vaccines

Virus-like particles (VLPs) represent a novel type of subunit vaccines that mimic the structure of live virus particles. VLPs are macromolecular structures derived from the self-assembly of viral structural proteins and are devoid of the viral genetic material; hence, they are non-infectious. Since viral antigens are present in their natural configuration, VLPs have the ability to stimulate the potent humoral and cellular immune responses relevant for protection compared to other traditional vaccine platforms. A VLP-based vaccine system has the potential to deliver multiple vaccine antigens to broaden the vaccine coverage.

Due to their high efficacy, ease of large-scale production, and improved safety compared to attenuated vaccines, VLPs are being extensively explored as vaccine candidates for a number of infectious diseases including human immunodeficiency virus (HIV), influenza virus, Ebola and Marburg viruses, West Nile virus, and enterovirus 71 (89,90). VLP-based vaccines against two viruses, hepatitis B virus (HBV) and human papillomavirus (HPV), are currently on the market. Recombivax-HB and Engerix-B, the two HBV vaccines produced using recombinant DNA technology in yeast cells, were both licensed by the Food and Drug Administration in 1986. Also produced in yeast cells and baculovirus vector-infected insect cells, the HPV vaccines—Gardasil and Cervarix—were approved in 2006 and 2009, respectively (90). These HBV and HPV VLP-based vaccines have been shown to have an excellent safety profile and induce long-lasting immunity. In addition to the use of VLPs as vaccine candidates against a target agent, they are being utilized to display a wide range of target molecules including self-antigens, peptide epitopes from pathogens, and non-protein targets such as carbohydrates or chemical agents. These adaptations are being explored in pre-clinical and clinical trials (91–96). Several VLP-based vaccines displaying self-antigens have been shown to greatly enhance immunogenicity against self-antigens in the treatment of cancer, autoimmune diseases, and HIV infection (92,93). Chemical-conjugated VLPs are being used to generate anti-smoking vaccines by linking nicotine to VLPs (96). Overall, the VLP-based vaccine platform looks promising for vaccine delivery for both infectious and noninfectious disease problems. Information on the currently available VLPs-based vaccines for human use is listed in Table 3.

DNA Vaccines

A plasmid DNA containing a gene under the control of a heterologous promoter can be used as a vaccine for a mammalian host. The introduced DNA is taken up by the host cells, and the gene of interest is expressed. The cells expressing the foreign antigen are recognized by the host immune system, leading to humoral and cell-mediated immune responses against the expressed protein. DNA vaccines have also been called polynucleotide vaccines or

Table 4 DNA vaccines for veterinary use

Target pathogen/ cancer	Delivery vector	Target antigen	References
West Nile Virus	DNA	preM and Env	(134)
Infectious hematopoietic necrosis virus	DNA	Glycoprotein	(135)
Canine melanoma	DNA	Human tyrosinase	(136,137)

AQ1

nucleic acid (NA) vaccines (Table 4). Such vaccines appear to have the advantages of both attenuated and inactivated vaccines but without their known limitations. NA vaccines elicit an immune response similar to that obtained with live attenuated vaccines. They also provide safety similar to that of inactivated vaccines, however, without the obvious side effects of adjuvants or animal-derived proteins.

The concept of a DNA vaccine evolved from initial studies in experimental animals in which inoculation with naked plasmid DNA resulted in a protective immune response (97). The drawback appears to be that after inoculation into a muscle, the efficiency of cellular uptake of the naked DNA is poor, and a large portion of the DNA is degraded before it reaches the nucleus for transcription. To increase the efficiency of DNA uptake by host cells and to reduce DNA degradation within the cell, a number of delivery systems, such as bombardment with gold nanoparticles coated with NA (98,99), incorporation of NA into liposomes and other polycationic lipids (100,101), biological erodable polymers (102), nanoparticles (103–105), electroporation (106–108), and others have been developed (Fig. 2). Alginate microspheres can be used for the encapsulation, delivery, and expression of plasmid DNA (109). Inoculation of mice with microspheres containing both plasmid DNA and bovine adenovirus type 3 (BAd3) resulted in a significant increase in transgene expression compared with those inoculated with microspheres containing only the plasmid DNA. As with other delivery systems, alginate microspheres led to a stronger mucosal or systemic immune response, depending on route of inoculation (110). Because alginate microspheres are most likely taken up by macrophages and dendritic cells, it may have a positive effect on the type of immune response elicited.

A number of factors that have an impact on the level and type of immune response produced by an NA vaccine include the type of immunogen, the dosage and number of inoculations, the heterologous regulatory sequences, the delivery system, the route of inoculation, and the presence or absence of immunomodulatory molecules. A variety of immunogenic antigens including HIV-1, Simian immunodeficiency virus (SIV), Human T-lymphotropic virus Type I, influenza virus, HBV, hepatitis C virus, herpes virus, Ebola virus, West Nile virus, Nipah Virus, Dengue virus, *Mycobacterium tuberculosis*, *Leishmania*, malaria, and many more have been expressed by NA vaccines and have demonstrated encouraging results (111–115).

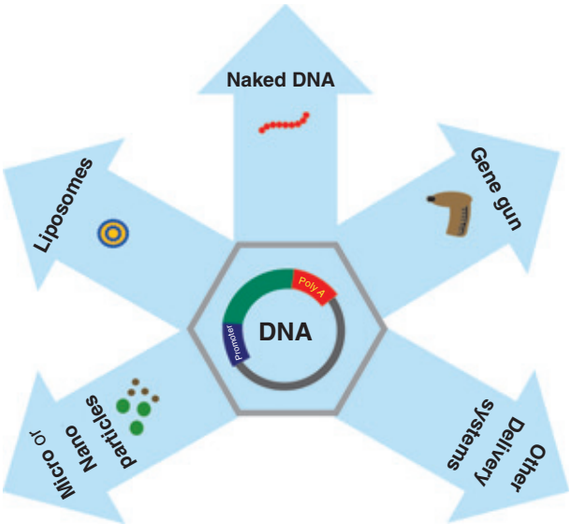

Figure 2 Various delivery approaches for nucleic acid vaccines.

Vectored Vaccines

Viral Vector-Based Vaccines

The route of vaccine delivery also plays an important role in determining the type of resultant immunity induced. For the development of an effective vaccine strategy for protection against mucosal pathogens such as respiratory and enteric viruses, a vaccine delivery system that can induce a protective mucosal immunity in the form of secretory IgA antibody, in addition to a systemic immune response, is extremely important. A number of viruses such as adenoviruses, pox viruses, herpes viruses, picornaviruses, togaviruses, orthomyxoviruses, paramyxoviruses, and others have demonstrated considerable potential as vectors for antigen delivery (116–120). Each virus vector system has its own unique qualities. A wide variety of foreign viral antigens of HIV-1, SIV-1, influenza virus, Ebola virus, Dengue virus, West Nile virus, herpes virus, hepatitis A virus, HBV, Hepatitis C virus, and others have been expressed in viral vectors, and vaccination-challenge studies in experimental animals have demonstrated moderate to complete protection (121–125). Immunization with such vectors leads to foreign viral antigen expression similar to that of natural infection without causing disease. Antigenic peptides are processed and presented on the cell surface with major histocompatibility (MHC) class I or class II antigens, and thus result in both humoral and cytotoxic T-cell responses. Immunogenic foreign epitopes can also be expressed on the virus surface by modifying the viral capsid or envelope protein (126). More than one foreign antigen can be expressed in the same vector to provide either broad protection or protection against a number of agents by inoculation with a single vector. A disadvantage is that many viral and bacterial vector-based vaccines may require a cold chain that may not be readily accessible in many developing countries (127).

Table 5 Viral vectored vaccines for veterinary use

Target pathogen	Delivery vector	Target antigen	References
Feline leukemia virus	Canarypox virus	Envelope protein (Env), group-specific protein (gag), polymerase (pol)	(65,66)
West Nile Virus	Canarypox virus	Protein M precursor (preM) and Env	(67,68)
Equine influenza virus	Canarypox virus	Hemagglutinin (HA)	(69,70)
Feline rabies virus	Canarypox virus	Glycoprotein G	(71–73)
Canine distemper virus	Canarypox Vector	HA and fusion antigen (FA)	(74)
Avian influenza (AI) virus	Fowlpox virus (FPV)	HA	(75)
Newcastle disease virus (NDV)	FPV	HA, neuraminidase, and FA	(76)
Rabies virus	Vaccinia virus	Glycoprotein G	(77)
AI and ND	NDV	HA	(78)
Infectious bursal disease virus & Mareks disease virus	Turkey herpes virus	Viral protein 2	(79)

Some of the most commonly used vectors are comprised of adenoviruses and poxviruses. Both adenovirus- and poxvirus-based vectors have a number of common advantages including (i) they are nonpathogenic for the host species; (ii) they include a wide host range; (iii) they are relatively thermostable; (iv) they have large capacity for foreign DNA insertion; (v) their vector construction is easy; and (vi) they have relatively high levels of foreign protein expression.

The vaccinia virus expressing rabies glycoprotein is licensed for use to control rabies in the wildlife population, especially raccoons, foxes, skunks, and coyotes (128). Baits containing a live vaccinia–rabies glycoprotein recombinant virus vaccine is distributed in the rabies endemic area with the intention that rabies-susceptible wild animals will eat these baits and become immunized against rabies virus (129). This approach has demonstrated satisfactory results. Taking advantage of the fact that avian pox viruses grow normally in avian cells, but result in an abortive infection in mammalian hosts; dogs and cats are immunized with an avipox–rabies glycoprotein recombinant for protection against rabies virus infection (130) and other pathogens.

To increase the safety of viral vectors for immunocompromised hosts and to control their indiscriminate spread, replication defective viral vectors have been developed. These vectors can be grown to high titers *in vitro*, but they are defective for *in vivo* replication. Replication-defective vectors undergo an abortive infection in an inoculated host leading to foreign antigen expression similar to replication-competent vectors. Replication-defective adenovirus vectors are generated by deleting the early region 1 (E1) genes (120,122,131,132). E1-deleted vectors can be grown in an E1-complementing cell line, and animals immunized with such vectors elicit a protective immune response (133). Several viral vector-based vaccines are already in the market for veterinary applications (Table 5).

Bacterial Vector-Based Vaccines

Similar to viral expression vectors, attenuated bacteria can be developed as vectors for foreign gene expression and delivery for bacterial vector-based vaccines. Immunogenic foreign epitopes can be expressed on bacterial surfaces by modifying cell surface proteins, fimbria, or flagella. There are many examples of bacterial vectors being investigated and used.

It has been demonstrated that the *Mycobacterium bovis* Bacille Calmette–Guérin (BCG) strain induces both strong humoral and cell-mediated immunity; therefore, it has been developed as a delivery vector with the assumption that the foreign proteins expressed by *M. bovis* in inoculated individuals will also raise a strong protective immune response (138). Brucella has also shown excellent potential as a vaccine delivery system (139). Since *Salmonella* and *Vibrio* colonize in the intestinal tract, attenuated strains of these bacteria have been developed as vectors for mucosal delivery (140–143). Various bacterial vectors have been used to express a number of bacterial (*Bordetella pertussis, Streptococcus pneumoniae, Yersinia pestis, and Listeria monocytogenes*), viral (herpes virus, influenza virus, HIV, SIV, and HBV), and parasitic (*Schistosoma mansoni and Leishmania major*) antigens (143). Significant improvements in the attenuation of bacteria, the stability, localization, and expression levels of heterologous antigens are required to market bacterial vector-based vaccines for use in humans or animals.

One improvement to bacterial vectors has been to enhance foreign gene expression by developing "balanced lethal" plasmid-based expression vehicles (144). A foreign antigen may form inclusion bodies or localize in the intracellular compartment of the vector, thereby affecting the type, levels, and duration of immune response elicited against the antigen. The *E. coli* α-hemolysin secretion system (HSS) that includes *HlyB*, *HlyD*, and *TolC* is involved in exporting the fused foreign antigens to extracellular compartment (145).

Using the HSS system for attenuated *Shigella dysenteriae*, the expression and secretion of Shiga toxin-B subunit were obtained (146).

ADJUVANTS

Adjuvants are substances that, when combined with vaccine antigens, enhance the magnitude and quality of the immune response. The inclusion of adjuvants can accomplish several goals. They can reduce the amount of antigen needed to induce a protective immune response (dose-sparing effect), increase the percentage of people or animals that produce an adequate response, and induce a longer-lasting immune response resulting in the decreased frequency of booster vaccinations. Adjuvants may also cause qualitative changes in the immune response. Such changes can include the induction of more effective T-cell responses by stimulating the differentiation of helper T (TH) cells into TH1 or TH17 effector cells, and the processing of antigens resulting in induction of CD8 T-cell responses, thereby enhancing the breadth of the immune response. Adjuvants that are currently used in licensed vaccines were developed empirically, and their mode of action is still poorly understood. Advances in immunology, particularly in our knowledge of the innate immune system, have begun to reveal possible mechanisms.

Immunostimulation

Activation of the adaptive immune response depends on signals from the innate immune system with a key role provided by dendritic cells. Immature dendritic cells that reside in non-lymphoid tissues such as the subcutis and skeletal muscle have the ability to take up vaccine antigens, migrate to the draining lymph node, and process and present antigens via MHC molecules to CD4 and CD8 T-cells. This provides a necessary, but not sufficient, signal (signal 1) for the activation of T-cells. T-cell activation requires a second signal provided by costimulatory molecules that are expressed on mature dendritic cells. The maturation of dendritic cells results in the expression of costimulatory molecules and is induced through activation of pattern-recognition receptors (PRRs). These PRRs are germline-encoded receptors for conserved microbial molecules such as lipopolysaccharide (LPS), peptidoglycans, flagellin, viral double-stranded RNA, and hypomethylated DNA, and include Toll-like receptors (TLRs), Nod-like receptors, and C-type lectins. The engagement of PRRs in dendritic cells also induces the synthesis and secretion of cytokines (signal 3) that drive the differentiation of CD4 T-cells to TH effector cells including TH1, TH2, and TH17 cells (147). Thus, dendritic cells not only present antigenic peptides to T-cells, but also convey information that determines the type of immune response which ensues based on the particular combination of PRRs that are activated. The PRRs are not only activated by microbial molecules, but also by "danger" molecules released from injured or necrotic cells. These include high-mobility protein group 1 (HMGB1), heat-shock proteins, and uric acid which, upon release, forms crystals that activate the NACHT, LRR and PYD domains-containing protein 3 (NALP3), one of the Nod-like receptors (148). NALP3 is the sensing component of an inflammasome, a multi-protein complex that includes caspase-1. Caspase-1 is important for the secretion of Interleukin (IL)-1 since IL-1 is first synthesized in an inactive pro-IL-1 form that accumulates in the cytoplasm of cells. Ligand binding by NALP3 causes activation of the inflammasome and activation of caspase-1 which cleaves pro-IL-1 resulting in the secretion of IL-1, a major pro-inflammatory cytokine (148). The expanding knowledge of PRRs is being applied to the design of adjuvants that can activate specific signaling pathways in dendritic cells resulting in appropriate and effective immune responses to the vaccine antigens. However, most adjuvants that are currently used in licensed vaccines do not contain microbial molecules, and direct activation of PRRs plays a limited role. Instead, tissue damage at the injection site and release of danger molecules that subsequently activate PRRs is probably a major contributing factor to the immunostimulating effect of these adjuvants.

Aluminum-Containing Adjuvants

The two main types of aluminum-containing adjuvants in vaccines are aluminum oxyhydroxide generally referred to as aluminum hydroxide (AH) and aluminum hydroxyphosphate referred to as aluminum phosphate (AP) (149). Both adjuvants are composed of primary nanoparticles that form loose aggregates with a large adsorptive capacity. The surface of AH contains primarily hydroxyl groups resulting in a high isoelectric point of 11.4 and a positive surface charge at neutral pH. In contrast, AP is a non-stoichiometric compound with both phosphate and hydroxyl groups at its surface. The ratio of phosphate to hydroxyl groups is determined by the conditions during the manufacture of AP, and this determines the isoelectric point. The isoelectric point of commercial AP varies between 4.5 and 5.5, giving it a negative surface charge at neutral pH.

Antigens adsorb to aluminum adjuvants via electrostatic interactions, ligand exchange, and hydrophobic interactions (149). Electrostatic interactions result from opposite charges of the antigen and the adjuvant surface. At neutral pH, proteins with an isoelectric point less than 7, are negatively charged and will adsorb to AH, but not to AP. On the other hand, basic proteins adsorb electrostatically to AP, but not to AH at neutral pH. Ligand exchange is the strongest adsorption mechanism in which phosphorylated antigens bind to aluminum adjuvants by exchanging a surface hydroxyl group for a terminal phosphate group. Since AH contains more surface hydroxyl groups than AP, phosphorylated antigens bind strongly to AH and weakly to AP (150).

Aluminum adjuvants are commonly used in veterinary and human vaccines. They effectively enhance the antibody response, have an excellent safety record, and are cheap. Vaccines formulated with aluminum adjuvants induce primarily a TH2 response in commonly used inbred strains of mice, but they induce a mixed TH1 and TH2 response in humans. Although it is generally thought that vaccines formulated with aluminum adjuvants fail to induce CD8 T cell responses, several studies suggest that

antigen-specific CD8 T cells are primed, but cytolytic responses are either weak or undetectable. (151–153). Additional signals such as stimulation through TLR4 are necessary to induce fully functional cytolytic CD8 T cells (153).

The mechanism by which aluminum adjuvants enhance the immune response is incompletely understood. Based on early experiments by Glenny et al. (154), it was postulated that aluminum adjuvants adsorb antigens and slowly release these from the injection site (depot theory). This theory led to recommendations that vaccines be formulated in such way that a significant proportion of antigens are adsorbed onto the aluminum adjuvants. However, vaccines formulated with aluminum adjuvants in which the antigens are not adsorbed, are also effective in enhancing the immune response (155). The non-adsorbed antigens diffuse from the injection site at the same rate as soluble antigens without aluminum adjuvants that are considered poorly immunogenic. This indicates that the immunostimulatory effect of aluminum adjuvants is not dependent upon the increased retention of antigen at the injection site (156).

Aluminum adjuvants induce tissue damage and a localized inflammatory response at the injection site (157). The release of chemokines and cytokines results in recruitment of inflammatory cells, first neutrophils and later macrophages and eosinophils. Although the exact mechanisms remain to be determined, it has been shown that aluminum adjuvants induce the release of uric acid, an endogenous danger signal. Treatment of mice with uricase diminished the immune response to antigens administered with aluminum adjuvants (158). Aluminum adjuvants also have a direct effect on dendritic cells and induce the release of IL-1 and IL-18 in a caspase-1–dependent manner (159,160). The NALP3 inflammasome has a critical role *in vitro,* but the *in vivo* role is controversial (161–163). Recent experiments suggest that both uric acid and aluminum adjuvant particles interact with cell membranes, resulting in lipid sorting and activation of dendritic cells independent of a specific receptor (164,165).

ASO4

ASO4 is an adjuvant system developed by GlaxoSmith-Kline and consists of aluminum hydroxide adjuvant with monophosphoryl lipid A (MPLA) (166). MPLA is derived from the lipid A component of LPS and, like LPS, stimulates the TLR4, but is much less toxic then LPS. The reduced toxicity of MPLA compared with LPS is attributed to a different manner of activation of TLR4 by MPLA versus LPS resulting in more limited cytokine secretion (167). The ASO4 adjuvant induces a more robust immune response with stimulation of both TH1 and TH2 cytokines compared with aluminum hydroxide adjuvant alone (168).

MF-59

MF-59 is an oil-in-water adjuvant developed by Novartis that is used in influenza vaccines and has been demonstrated to be effective in combination with various other vaccine antigens (169). Similar to aluminum adjuvants, MF-59 induces a TH2-biased immune response. There is little or no evidence that MF-59 induces activation of CD8 T-cells. The exact mechanism by which MF-59 enhances the immune response is not well understood. Studies in genetically engineered mice indicate that the response to influenza vaccines formulated with MF-59 is independent of NALP3 and caspase-1 (24). However, the immune response is reduced in mice that lack ASC, a protein that is a component of the inflammasome, but in this case appears to have an inflammasome-independent role in response to MF-59 (170). MF-59 induces inflammation at the injection site with increased expression of chemokines and cytokines and infiltration with neutrophils, macrophages, and dendritic cells (171,172). This leads to phagocytosis of antigens by all cell types and migration to the draining lymph nodes (172).

Saponins

Saponins of the bark of the Quillaja saponaria tree have long been known to have immunostimulatory activity. A partially purified fraction, Quil A, has reduced toxicity, stimulates more potent adjuvant activity and is used in veterinary vaccines. Quil A can be further fractionated into fractions having different degrees of toxicity. QS-21 is a less toxic component with strong adjuvant activity. Saponins stimulate both the humoral (primarily IgG2a antibodies in the mouse) and cell-mediated immune responses. QS-21 causes protein antigens to be processed and presented via the MHC I pathway, resulting in cytotoxic T-cell responses. Quil A stimulates the secretion of IL-1β by peripheral blood mononuclear cells *in vitro*, in a caspase-1 and NALP3-inflammasome–dependent fashion similar to aluminum adjuvants (16). However, the role of inflammasome activation in the immunostimulatory effect of Quil A *in vivo* remains to be determined.

Immune-stimulating complexes (ISCOMs) are particles of size 30–40 nm consisting of Quil A, phospholipids, cholesterol, and antigen (27). They are used in a commercial vaccine for equine influenza. A related adjuvant is ISCOMATRIX™, which comprises cholesterol, phospholipids, and saponins. It can be mixed with antigens, making it more versatile since the antigens do not have to be embedded in the lipid particles. ISCOM and ISCOMATRIX™-adjuvanted vaccines stimulate a strong humoral and cell-mediated immune response caused by the immunostimulatory actions of Quil A and targeting of the particles to macrophages (173).

ROUTES OF VACCINE DELIVERY

Parenteral Vs. Mucosal Route

The success of vaccination depends on the efficiency of antigen presentation to the host immune system. Depending on the type of antigens, a parenteral or systemic (such as intramuscular, subcutaneous, intraperitoneal, intradermal, or even intravenous) or mucosal (such as intranasal, oral, intrarectal, intravaginal, or ocular) route of inoculation is used to induce an appropriate level or type of immune response. For some pathogens, immunization via the mucosal route results

in better IgA production, leading to more effective mucosal immunity.

Parenteral Route

The parenteral route of immunization still remains the major route of choice for the majority of currently available vaccines mainly due to the type of antigens and correlates of protection. Millions of people receive inactivated influenza vaccine by parenteral administration every year. The immune response, i.e., the increase in the number of influenza virus-specific antibody-secreting cells in peripheral blood and tonsils, increase rapidly to reach a peak within one week after vaccination (174). For many other antigens, however, the usefulness of parenteral vaccination is limited by the insufficient induction of mucosal immune responses. An exception has been the parenteral vaccination of a DNA vaccine encoding glycoprotein D of herpes simplex virus type 2, which resulted in systemic cellular and humoral responses. The mucosal antibody response generated by intramuscular or intradermal vaccination was comparable with that obtained by mucosal vaccination. The DNA vaccine was able to stimulate a response in the Peyer's patches, a major inductive site for mucosal responses (175).

Parenteral vaccination is not practical in developing countries for large-scale vaccination since it requires the use of hypodermic needles and trained healthcare personnel. This problem becomes even more significant for veterinary vaccines. Advances in needleless injectable systems have made the parenteral vaccination easier, but it still requires individual handling. Examples of needleless injection systems are PowderJect®, Medi-Jector®, Biojector®, Vitajet®, Bio-Set®, and Intraject®. They all use high pressure released in a very short period to deliver drugs through the skin. A jet-immunization technique was used for intraoral administration of DNA in the cheek, resulting in high IgA mucosal responses (176). The intraoral jet-injection technique for DNA vaccine delivery has the advantages of being a simple and rapid way to administer the DNA in solution and to provoke specific mucosal IgA after administration in the mucosal-associated lymphoid tissue.

The results of parenteral vaccination depend on the route of administration. For plasmid DNA vaccines, the highest levels of antibodies were induced by intramuscular and intravenous injections, although significant titers were also obtained with sublingual and intradermal delivery (177). Delivery to the skin by the gene gun induced exclusively IgG1 antibodies (TH2-like) at four weeks and only very low IgG2a levels at later times. Other routes, such as intraperitoneal or subcutaneous did not result in significant immune responses for the DNA plasmid vaccines.

Dual-Chamber Syringe

A dual-chamber syringe delivery system can be used for the delivery of two established vaccines (e.g., diphtheria–tetanus–pertussis vaccine and inactivated poliovirus vaccine) at the same time. The proximal chamber contains a vaccine in the freeze-dried solid state, and the distal chamber contains a vaccine in the liquid formulation that allows reconstitution of the vaccine in the proximal chamber. The immune response by the dual-chamber delivery of vaccination was equivalent to that by the separate-injection method of vaccination. The dual-chamber syringe can be used for safe and effective delivery of two different vaccines that are not yet available as a single formulation for pediatric applications (178). The primary advantage of the dual-chamber syringe is that it reduces the cost of vaccine delivery and, at the same time, increases vaccine acceptability and rate of coverage (179).

Transdermal or Transcutaneous Route

This route of immunization is attractive because it does not require the specially trained personnel necessary for needle injections. Topical application of antigens to intact skin has shown promising results for the administration of DNA-based vaccines. Noninvasive gene delivery by pipetting adenovirus- or liposome-complexed plasmid DNA onto the outer layer of skin achieved localized transgene expression within a restricted subset of skin in mice. It also elicited an immune response against the protein encoded by the DNA (180).

For improved results, the feasibility of non- adjuvant, needle-free skin immunization by transdermal electroporation has been explored (181). The transdermal electroporation route elicited higher responses to a myristoylated peptide than did intradermal immunization. For diphtheria toxoid, however, the result was the opposite. It appears that transdermal electroporation is a promising technique for non-adjuvant skin immunization, especially for low molecular weight, weakly immunogenic antigens. Topical application of antigen and cholera toxin or bacterial exotoxin to the skin surface resulted in detectable antigen-specific IgG in plasma and mucosal secretions (182,183). Transcutaneous immunization appears to induce potent, protective immune responses to both systemic and mucosal challenge (184). Transcutaneous immunization has also been attempted utilizing the microneedle patches. Microneedles are small enough not to cause any pain but large enough to penetrate the skin to deliver antigen to dermis for higher efficacy (185–187).

Mucosal Route

Vaccination through mucosal routes provides the unique advantage of mucosal immunity that may not be obtained through parenteral immunization. Vaccination by mucosal route provides a number of advantages over parenteral vaccination: (i) mucosal vaccination does not involve hypodermic needles which are not user-friendly; (ii) the total surface area of the mucosal surfaces in the gastrointestinal (GI), respiratory, and urogenital tracts where the number of infectious pathogens coming into contact with the host is huge; and (iii) the prevention of infections at the mucosal surface provides an immunological first line of defense against diseases (7). Parenteral vaccination is quite often insufficient in inducing mucosal immune responses because stimulation of the MALT usually requires direct contact between the immunogen and the mucosal surface (188). The mucosal tissues are protected by an interconnected local immune system,

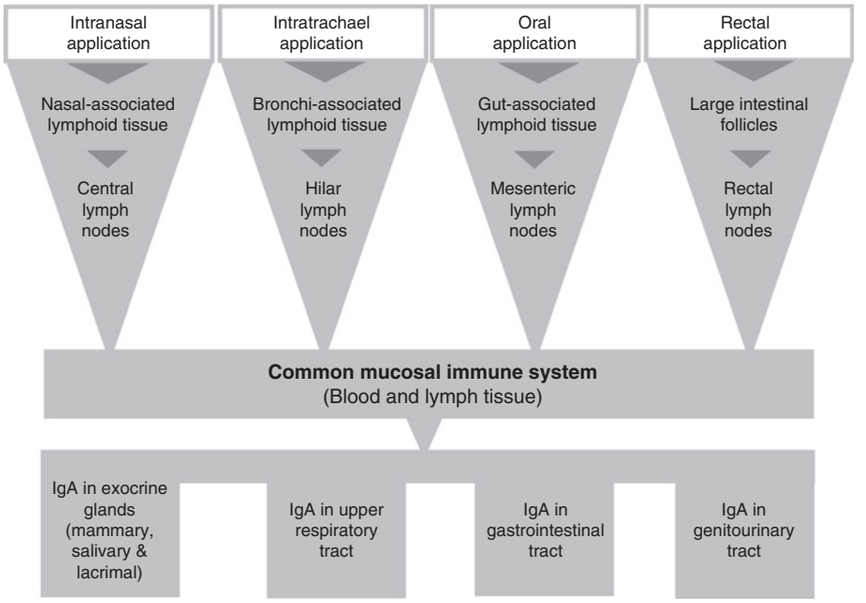

Figure 3 Mucosal immunization leading to dispersal of IgA antibodies to common mucosal immune system.

which is essentially separated from the systemic immunity (189). In a common mucosal-defense system, an antigen interacting with localized lymphoid tissue can stimulate IgA precursor cells that may then migrate to other mucosal surfaces to elicit immune response in other mucosal tissues. It is known that the mucosal immune system produces 70% of the body's antibodies (190) Fig. 3 shows a schematic description of the common mucosal-immunization system. Mucosal delivery of numerous antigens by a variety of routes (oral, nasal, tracheal, and rectal) has been shown to elicit immunity at mucosal surfaces mediated by secretory IgA. The presence of MALT indicates that mucosal vaccination at a certain site in the body can be achieved by mucosal immunization at the distal site of the body. Although the mucosal and systemic humoral immune systems function essentially independent of each other, an antigen administered by one route can modify responsiveness to subsequent immunization by an alternate route (191).

Oral Route

Oral vaccination is the preferable mode of vaccination because of ease of use and low cost of manufacturing (192). Furthermore, the GI tract provides the largest component of the mucosal immune system that has been well-characterized. After oral vaccination, an antigen, which is typically loaded in microspheres, is taken up by M-cells in the Peyer's patch of the gut-associated lymphoid tissue. The antigen is then passed to dendritic cells, macrophages, and B cells. These cells, in turn, present the antigen to T-helper lymphocytes. The lymphocytes migrate into the blood via the mesenteric lymph nodes and the thoracic duct. These cells subsequently localize in the effector sites, i.e., mucosal membranes of the GI tract, upper respiratory tract, genitourinary tract, and glandular tissue. At the effector sites, the migrating B-cells develop into plasma cells that produce IgA antibodies. Polymeric IgA is then released as secretory IgA (sIgA) through epithelial cells.

One of the limitations of oral vaccination is that it does not always induce sufficient immunity. Because most antigens are proteins in nature, they may be degraded by enzymes in the GI tract as well as by acids in the stomach. This is why soluble antigens administered orally are not effective. Thus, prevention of the antigen degradation is the first step toward successful oral vaccination. Adding protease inhibitors before oral vaccination may induce complete immunity, but this approach is not practical. There are many different enzymes that may not be inhibited by a particular protease inhibitor, and, more importantly, the action of protease inhibitors may not occur at the same time that the antigens are present in the GI tract. Second, the systemic uptake of antigens from the GI tract is poor. Even after oral intake of one gram of antigen, only a nanogram range of antigenic material was found to pass the intestinal barrier (193). It is also possible that for certain antigens, oral vaccination may simply be less effective than parenteral vaccination in inducing systemic immunity (194). The protection resulting from oral vaccination is known to last for a relatively short period, ranging from a few months to one year. To obtain the desirable immunity equivalent to systemic immunization, oral vaccination requires much higher and more frequent oral doses. The use of highly effective adjuvants in oral vaccine formulation may result in strong and long-lasting immunity in mucosal tissues.

The issues of degradation of antigens in the GI tract and the poor systemic uptake of antigens from the GI tract have led to encapsulation of antigens in microparticles or nanoparticles to protect the antigen(s) from degradation and to allow better antigen uptake by the M-cells in the Peyer's patches.

A large number of studies have shown that antigens orally delivered in microparticles resulted in good mucosal immunity (191,195–197). Successful oral vaccination relies on targeting of microparticles to the Peyer's patches. It is known that the surface chemistry of microparticles affects the targeting to and uptake by M-cells in the Peyer's patches (198). The exact relationships between the surface chemistry and the uptake by Peyer's patches, however, have not been fully understood. Interestingly, a virus can be regarded as a particulate vaccine delivery system. Many viruses are highly effective in inducing immunization after oral vaccination. Norwalk virus, which is a major cause of epidemic gastroenteritis, was immunogenic in healthy human adults even when administered without adjuvants (199). Influenza virus can also elicit immune response after oral administration.

Intranasal Route

This non-invasive immunization route has been quite effective for various vaccine delivery systems. Immunization of mice with tetanus toxoid, in both solution and microsphere-encapsulated formulations, has resulted in high levels of specific IgG and IgA antibodies (200). Nasal vaccine delivery is known to be superior to oral delivery in inducing specific IgA and IgG antibody responses in the upper respiratory tract (201). Nasal immunization is also known to be preferable to the oral route for distant mucosal vaccination that might be used to prevent adhesion of pathogens to the urogenital tract (201). It is interesting to note that the volume of the nasally instilled vaccine is important (200). The larger volume (e.g., 50 mL) of microsphere suspension resulted in a higher percentage of particles entering the lungs than did the lower volume (e.g., 10 mL) instillation. It is generally believed that microspheres that adhere to the nasal mucus elicit better immune response, and, for this reason, many microspheres that have been used extensively in the preparation of nasal vaccine formulations are made of mucoadhesive polymers (202). The first nasal spray formulation containing live attenuated influenza virus, FluMist®, was first approved in 2003 (203).

Pulmonary vaccination is especially useful in mass vaccination campaigns. A conventional method of pulmonary delivery of drugs using metered-dose, propellant-driven, small-particle aerosols was used to deliver killed whole bacterium vaccines. The results showed good stimulation of mucosal immunity against respiratory infections in animals (204). Advances in powder inhaler devices have made it possible to deliver vaccines via the pulmonary route using dry powder inhalation technologies (205). Dry powder vaccine in the size range of 1–5 μm in diameter is used for the maximum alveolar (deep lung) deposition (204). Direct gene transfer into the respiratory system can be carried out for either therapeutic or immunization purposes. Cells in the lung can take up and express plasmid DNA whether it is administered in naked form or formulated with cationic liposomes. For a given dose of DNA, the results can be improved when the DNA is mixed with the minimum amount of lipid that can complex it completely (206). Such a complex formation can be considered a formation of microparticles that can enhance cellular uptake and subsequent immune responses.

The combination of mucosal and systemic immunization routes (e.g., parenteral immunization followed by oral immunization or vice versa) can induce mucosal immune responses that are superior to immunization by either route alone (194). Pigs showed protection after intramuscular inoculation with a formalin-inactivated *Mycoplasma hyopneumoniae* vaccine in incomplete Freund's adjuvant, followed by a booster inoculation with the same vaccine in microspheres onto the mucosal surface of Peyer's patches by a surgical procedure (207).

ANTIGEN DELIVERY SYSTEMS

The main goal of antigen delivery systems is to present antigens to elicit an efficient immune response *in vivo* and to maintain the antigens in stable form during storage. It is necessary to develop vaccine formulations that would preserve the antigen and deliver it to a specific target organ over a desired period. Continuous or multiple pulsatile release of vaccine during the desired period would eliminate the need to administer multiple doses of a vaccine for obtaining satisfactory immune responses (208). The antigen delivery system plays a significant role in inducing the level and type of immune responses.

As described earlier in this chapter, live attenuated vaccines are relatively easy and cheap to manufacture because they do not require purification of antigens or formulation with adjuvants (7). Due to the success of live attenuated vaccines, live attenuated bacteria and viruses have been developed as a delivery system for foreign antigens to induce protective immune responses. The viral and bacterial delivery systems have the advantage that they can be administered by the natural route of infection. Information on the viral and bacterial delivery systems is presented earlier in this chapter.

PARTICULATE DELIVERY SYSTEMS

Inactivated or subunit vaccines generally result in immune responses of lesser magnitude and of shorter duration compared to live attenuated vaccines. In order to protect antigens and to improve their cellular uptake, adjuvants or delivery systems are needed. Particulate antigen delivery systems include polymeric microparticles, liposomes, VLPs, neosomes, cross-linked protein crystals, and polymeric nanoparticles. The definition of microparticles should be broad enough to include all other forms, such as protein aggregates. The size of microparticles used in the vaccine is usually less than 50 μm (195). It is common, however, to call any particles less than a few hundred micrometers microparticles. For this reason, it is important to specify the average size of microparticles for particular applications, because their size often affects the vaccine efficacy. Polymeric microparticles and liposomes have been used extensively as controlled-release formulations for many drugs including antigens. They have been quite useful in the oral delivery of antigens because encapsulation in microparticles can protect antigens from acidic and enzymatic degradation in the GI tract. Particulate vaccine delivery systems show improved immune responses because of the protection of the loaded

antigens from degradation and their slow release. For this reason, particulate delivery systems are often considered adjuvants (209).

Polymer Microparticles

A large number of polymers, such as poly(methyl methacrylate), poly(butyl cyanoacrylate), poly(lactide-co-glycolide), polyarcyl-starch, dextran, albumin, and alginic acid, have been used for making microparticles for vaccine delivery. Polymers that have been used for controlled drug delivery can be used for vaccine delivery (210). Preparation of microparticles from water-insoluble polymers [e.g., poly(methyl methacrylate), poly(butyl cyanoacrylate), and poly (lactide-co-glycolide)] requires use of organic solvents or high temperature, both of which may not be good for maintaining tertiary structures of antigens. Preparation of water-soluble polymers frequently requires a cross-linking reaction to ensure that the polymers remain insoluble. It is possible that chemical cross-linking agents cross-link not only polymer chains, but also antigen molecules. Absorption of water into hydrophilic polymers results in swelling of the network, i.e., formation of hydrogels or aquagels. Preparation of microparticles from hydrophilic polymers, e.g., calcium cross-linked alginate microparticles (211), is preferred because it does not require organic solvents or high temperature. Polymers that have been used in immunization vary depending on the route of administration.

For parenteral vaccination, biodegradable polymeric microparticles made of poly(lactide-co-glycolide) are commonly used as vaccine carriers. Poly(lactide-co-glycolide) has been well-characterized and known to be highly biocompatible. The size of microparticles can be easily controlled, and microparticles of less than 100 μm in diameter can be easily administered by injection through standard-sized needles (22 gauge or smaller). Because of the slow degradation of the polymer, antigens are slowly released from the microparticles, resulting in enhanced immune responses. Other polymers, such as chitosan, have been used for preparation of vaccine formulations. Because one of the important roles that microparticles play in immunization is the slow release of antigens, a number of approaches have been tried to achieve antigen release at desired rates. The surface of microparticles can be modified to alter the adsorption and desorption kinetics of antigens. Alternatively, the pore size can be varied to control the release of antigens from microparticles.

Microparticles are known to enhance uptake by M-cells in the Peyer's patches, and the effectiveness of the uptake depends on the size of microparticles. It is generally thought that microparticles smaller than 10 μm are preferentially absorbed by M-cells, and the smaller the size, the better the absorption. One study using microparticles of different sizes showed that the efficiency of uptake of 100-nm particles by the intestinal tissue was 15- to 250-fold higher than that of larger sized microparticles (212). In addition to the small size, microparticles with more hydrophobic surface property are anticipated to be absorbed better than those with more hydrophilic surface property. Once microparticles are in the GI tract, adsorption of numerous proteins and polysaccharides present in the GI tract could alter the surface chemistry drastically, and thus, it is difficult to correlate a particular surface chemistry of the native microparticles with the absorption ability.

Liposomes

Liposomes are vesicles composed of naturally occurring or synthetic phopholipids. The bilayer structure can be single- or multi-compartment, and the size can vary from >1 μm to <10 μm. Following interaction of negatively charged lipid molecules with divalent cations, a solid, multi-layered, crystalline structure called cochleate is formed. Because liposomes and cochleates can protect antigens from the GI tract and deliver them to the Peyer's patches, they have been exploited as a delivery system for oral vaccination. The surface charge of liposomes is known to affect the immune responses. Positively charged liposomes containing soluble antigens were reported to function as a more potent inducer of antigen-specific, cytotoxic T-lymphocyte responses, and delayed-type hypersensitivity responses than negatively charged and neutral liposomes containing the same concentrations of antigens (213). Studies have showed that the positively charged liposomes delivered proteinaceous antigens efficiently into the cytoplasm of the macrophages/antigen-presenting cells where the antigens are processed and presented by MHC class I molecules to induce the cell-mediated immune response (213).

Fusogenic liposomes containing highly immunogenic glycoproteins of the Sendai virus on their surface showed enhanced antigen-specific humoral immunity in mice (214). The levels of anti-ovalbumin antibody were markedly increased in serum from mice immunized with ovalbumin-encapsulated in fusogenic liposomes. It appears that the fusogenic liposomes function as an immuno-adjuvant in inducing antigen-specific antibody production.

Virosomes

Virosomes are liposomes containing viral fusion proteins that allow efficient entry into cells' fusion with endosome membranes. Viral fusion proteins become activated in the low pH environment in the endosome to release its contents into the cytosol (215). Virosomes have potential in the design of combined vaccines targeted against multiple antigens from multiple pathogens (216). Hepatitis A and influenza vaccines constructed on virosomes elicited fewer local adverse reactions than did their classic counterparts and displayed enhanced immunogenicity. Virosome-formulated influenza vaccine has also been shown to be safe and immunogenic when administered by the intranasal route (217). Other studies have suggested that immunopotentiating virosomes can be a suitable delivery system for synthetic peptide vaccines.

VLP-Based Delivery System

VLPs as subunit vaccines have been described earlier in this chapter. VLPs are immunogenic molecules that allow for covalent coupling of the epitopes of interest (218). Parvovirus-like particles have been engineered to express

foreign polypeptides in certain positions resulting in the production of large quantities of highly immunogenic peptides and the induction of strong antibody, T-helper cell, and cytotoxic T-lymphocyte responses (218). Immunization of VLPs without adjuvants via the mucosal route is known to elicit specific antibodies at mucosal surfaces and also systemic VLP epitope–specific T-cell responses (219).

Micelles

Micelles are aggregates of detergent molecules in aqueous solution. Detergents are water-soluble, surface-active agents composed of a hydrophilic head group and a hydrophobic or lipophilic tail group. They can also align at aqueous/non-aqueous interfaces, reducing surface tension, increasing miscibility, and stabilizing emulsions. Polymeric micelles made of block copolymers such as poly(ethylene oxide)–poly(propylene oxide)–poly(ethylene oxide) have been used as a delivery system for hydrophobic drugs. They can also encapsulate antigens for vaccination.

Niosomes

Niosomes are non-ionic surfactant vesicles and can be used to develop a vaccine-delivery system by the oral route. Ovalbumin was encapsulated in various lyophilized niosome preparations consisting of sucrose esters, cholesterol, and dicetyl phosphate. Encapsulation of ovalbumin into niosomes consisting of 70% stearate sucrose ester and 30% palmitate sucrose ester (40% mono-, 60% di/triester) resulted in a significant increase in antibody titers in serum, saliva, and intestinal washings (220).

Cross-Linked Protein Crystals

Cross-linked protein crystals have been tested as antigens. The immunogenicity of cross-linked protein crystals of human serum albumin was 6- to 30-fold higher in antibody titer than that of the soluble protein (221). Cross-linked protein crystals appear to function as a depot from which there is a slow release of antigen(s). The cross-linked protein crystals present high stability, purity, and biodegradability, all of which are highly attractive features for vaccine formulation (221). Since cross-linked protein crystals are microparticulates, they can also be used for vaccination through various routes.

NANOPARTICLE DELIVERY SYSTEMS

On the surface, nanoparticles are different from microparticles only in size. But the size is not the only difference between the two, at least for vaccine delivery. Nanoparticles have been demonstrated to be superior to microparticles in targeted vaccine delivery (222). Dendritic cells were identified as key antigen-presenting cells instructing the immune system to which antigens the immune responses should be directed. Targeting antigens to surface receptors that are preferentially expressed by dendritic cells resulted in markedly enhanced antigenicity. Antigens were entrapped within nanoparticles, as well as microparticles, known to be taken up preferentially by antigen-presenting cells. Nanoparticles are known to have excellent tissue penetration, to access the lymphatics, and to drain freely to the lymph nodes where a large number of lymph node–resident dendritic cells exist. On the other hand, microparticles are known to remain at the injection site, requiring active transport by phagocytic cells to reach the lymph nodes (223). Thus, nanoparticles appear to be more favorable than microparticles for targeted vaccination.

IMMUNOMODULATION

Immunomodulation refers to treatments that alter immune responsiveness in a non-antigen-specific manner. Enhancement of the immune response is desired for enhanced response to a vaccine and in the treatment of chronic infectious diseases and neoplastic diseases. Suppression of the immune response is needed in cases of inappropriate or exaggerated immune response, including allergies and autoimmune diseases. There are a number of drugs that affect the activity of the immune system. The effect of currently available immunosuppressive drugs is very broad with undesirable side effects.

COSTIMULATION

Activation of T-cells requires the recognition of the MHC/peptide complex by the T-cell receptor followed by a costimulatory signal. The binding of CD28 on T-cells to B7 molecules on antigen-presenting cells serves as the major costimulatory signal.There are at least two B7 molecules, B7-1 (CD80), and B7-2 (CD86). Activation of antigen-presenting cells (e.g., dendritic cells) results in increased expression of B7-2, followed by B7-1. A second T-cell ligand of the B7 molecules is cytotoxic T-lymphocyte antigen-4 (CTLA-4 or CD152), which is expressed on both CD4+ and CD8+ T-cells after binding of the T-cell receptor to the MHC/peptide complex on antigen-presenting cells. In contrast to the positive signal provided by CD28 (224), CTLA-4 down-regulates T-cell responses. CTLA-4 has a higher affinity for the B7-molecules than does CD28 and may either prevent the activation of T-cells when B7 expression by dendritic cells is low and/or terminate the immune response when its expression is strongly increased. A soluble chimeric protein, CTLA-4Ig, blocks the binding of both CD28 and CTLA-4 to the B7 molecules, thus preventing T-cell activation. Administration of this protein to patients with psoriasis vulgaris, an immune-mediated skin disease, resulted in significant improvement in approximately 50% of the patients (225). Selective inhibition of CTLA-4 with specific antibodies may boost the immune system. The combination of surgery and anti-CTLA-4 antibody therapy was highly effective in the prevention of metastatic recurrence in a mouse prostatic carcinoma model (226). Other CD28 and B7 homologs continue to be identified and appear to play a role in costimulation.These molecules may provide additional targets for immunomodulation and suggest that it may be possible to fine-tune the immune response through pharmacologic intervention.

CPG DNA

There is a higher percentage of the preferentially methylated CpG oligonucleotides (CpG–ODN) in bacterial DNA

compared to vertebrate DNA. CpG–ODN stimulates the secretion of IL-12 by macrophages and dendritic cells, and thus provides a potent stimulus for type-1 immune responses (227). It also directly stimulates B-cells to proliferate and differentiate into immunoglobulin-secreting cells. The effect of CpG–ODN is through the activation of TLR9 (228). The DNA appears to enter the cell via endocytosis, and some of the DNA escapes the endosomes into the cytoplasm of the cell where it activates the TLR9 pathway.

Administration of CpG–ODN to mice protected against subsequent challenge with the intracellular bacteria *L. monocytogenes* and the intracellular protozoa *L. major*. In addition, the CpG–ODN cured established *L. major* infections. The strong type-1 immunostimulatory property of CpG–ODN makes this compound a good candidate for vaccine adjuvants. Indeed, coadministration of CpG–ODN with antigen markedly boosts the humoral and cell-mediated immune responses. Allergic diseases such as asthma and atopic dermatitis are caused by type-2 immune responses directed against otherwise innocuous antigens. Treatment with CpG–ODN cleared established disease in a mouse model of airway hyper-reactivity, suggesting a CpG-induced reversal to type-1 immune response. CpG DNA may also have a place in the immunotherapy of cancer because of its ability to activate NK cells through the induction of IL-12. Administration of CpG–ODN in combination with monoclonal antibodies directed against tumor antigens greatly enhanced the survival of mice that had been inoculated with tumor cells.

CYTOKINES

Cytokines play a vital role in the regulation of the immune and inflammatory response, and therefore are potential targets for therapy. Important limitations include the pleiotropy and redundancy in the cytokine system and the short half-life and short action range of most cytokines. In spite of these limitations, considerable effort is spent on developing reagents that either block or enhance the activity of a specific cytokine.Two remarkable successes of cytokine therapy are the treatment of multiple sclerosis with interferon-β and the treatment of rheumatoid arthritis and inflammatory bowel disease with tumor necrosis factor-α (TNF-α) inhibitors.

Interferon-β

Subcutaneous injections of recombinant or natural interferon-β reduces the rate of exacerbation of relapsing-remitting multiple sclerosis (229,230). Interferon-β reduces the production of TNF-α and increases the secretion of IL-10. TNF-α is a proinflammatory cytokine that may contribute to demyelination in multiple sclerosis. IL-10 suppresses macrophage function and the production of TNF-α. In addition, interferon-β may reduce the entry of leukocytes into the central nervous system, a critical component in the inflammation that causes the lesions in multiple sclerosis.

TNF-α Inhibitors

TNF-α is produced as a transmembrane precursor molecule by various cells in the body. It is cleaved by the TNF-α–converting enzyme and forms trimeric aggregates that bind to either the TNF-receptor (TNFR) I or TNFR II that are expressed on many different types of cells. Extracellular domains of the TNFR can be cleaved by enzymes and can inhibit TNF-α activity by preventing binding of TNF-α to cell-bound receptors. Recent studies have demonstrated that inhibition of TNF-α activity resulted in significant improvement of the clinical condition of many patients with rheumatoid arthritis and inflammatory bowel disease (231,232). These studies clearly demonstrate an important role of TNF-α in rheumatoid arthritis and inflammatory bowel disease, although the precise mechanisms remain to be determined. The inhibition of TNF-α activity is achieved by treatment with anti-TNF-α monoclonal antibodies or with soluble TNFR-fusion protein. To reduce the induction of antibodies against the mouse monoclonal antibodies, the monoclonal antibodies are chimeric (i.e., the constant portion is derived from human immunoglobulins and the TNF-α–specific variable portion is derived from mice) or humanized (all of the immunoglobulin is human except for the complementarity determining regions that fold into the TNF-α–binding region). The TNFR-fusion protein is constructed from the extracellular domain of TNFRII and the Fc portion of human immunoglobulins. This construct has a much longer half-life than the naturally occurring soluble TNFR.

FUTURE CHALLENGES IN VACCINE FORMULATIONS

Advancements in microbial pathogenesis, immunology, molecular biology, vaccine formulation, and expression technology have formed the foundation for a new generation of vaccines and other pharmaceutical products. New developments in the delivery system have provided novel ways to enhance the immunogenicity of subunit antigens or nucleic acids by their controlled release and reduced degradation. Because the majority of pathogens enter their hosts via mucosal routes, the new-generation vaccines should have the advantage of providing effective protection at the mucosal sites. An ideal vaccine would be one that provides life-long protection with a single inoculation. New-generation vaccine formulations should also have high stability, thus avoiding the problems commonly observed during storage. Theoretically, various controlled-release technologies can be used to release antigens over time in a sustained or pulsatile manner and to direct antigens to specific antigen-presenting cells for increased vaccine efficacy. In addition to controlled-release technology, the single-shot vaccination requires the development of better adjuvants. The mechanism of action of such adjuvants should be known so that reproducible results can be obtained in a mass vaccination program.

It will be a challenge to immunize immunocompromised, immature, older, or pregnant hosts against various vaccine-preventable diseases due to their unique immune status. In the past decades, new or previously unknown infectious agents have emerged; therefore, a versatile vaccine platform that can be easily adapted to a new or

emerging pathogen will greatly enhance our capability to protect human and animal health. Since the majority of new pathogens are emerging from animals, a concise global effort will be critical in controlling disease in the animal reservoir. In addition, the constant change of infectious agents under immune pressure may lead to escape mutants that are not protected by the available vaccines. The world population is growing at a fast pace, and an additional one billion people may be added every 10–15 years. Taking care of the health of all these individuals, knowing that the maximum population growth will be in the developing countries, will be a tremendous challenge. Of vaccines already in use, more efficient single-dose vaccines need to be developed with more attention to the cost and number of inoculations required. Vaccine production capacity and cost will determine the effectiveness of many vaccine programs. Development of broadly protective vaccines and multivalent vaccines will certainly be beneficial in the global efforts to control and, hopefully, even eradicate some of these infectious diseases.

ACKNOWLEDGEMENT

This work was supported by Public Health Service grant AI059374 from the National Institute of Allergy and Infectious Diseases. We are thankful to J. Kovach for her excellent secretarial assistance and S. V. Vemula for gathering information for the tables.

ARTICLES OF FURTHER INTEREST

Vaccine, p.
Adjuvant, p.
Pandemic, p.
Influenza, p.
Infectious Disease, p.

REFERENCES

1. Top FH Jr, Grossman RA, Bartelloni PJ, et al. Immunization with live types 7 and 4 adenovirus vaccines. I. safety, infectivity, antigenicity, and potency of adenovirus type 7 vaccine in humans. J Infect Dis 1971; 124: 148–54.
2. Neumann G, Watanabe T, Ito H, et al. Generation of influenza a viruses entirely from cloned cDNAs. Proc Natl Acad Sci USA 1999; 96: 9345–50.
3. Sabin AB, Boulger L. History of sabin attenuated polio-virus oral live vaccine strains. J Biol Stand 1973; 1: 115–18.
4. Parkman PD, Meyer HM Jr, Kirschstein RL, et al. Attenuated rubella virus. I. Development and laboratory characterization. N Engl J Med 1966; 275: 569–74.
5. Plotkin SA, Farquhar J, Katz M, et al. A new attenuated rubella virus grown in human fibroblasts: evidence for reduced nasopharyngeal excretion. Am J Epidemiol 1967; 86: 468–77.
6. Bunyak EB, Hilleman MR, Weiber RE, et al. Live attenuated rubella virus prepared in duck embryo cell curlture I. development and clinical testing. J Am Med Assoc 1968; 204: 195–200.
7. Roberts M, Chatfield SN, Dougan G. Salmonella as carriers of heterologous antigens. In: O'Hagan DT, ed. Novel Delivery Systems for Oral Vaccines. Ann Arbor, MI: CRC Press Inc, 1994: 27–58.
8. Ozaki T, Matsui T, Ichikawa T, et al. Clinical trial of the Oka strain of live attenuated varicella vaccine on healthy children. Biken J 1984; 27: 39–42.
9. Asano Y and Takahashi M. Clinical and serologic testing of a live varicella vaccine and two-year follow-up for immunity of the vaccinated children. Pediatrics 1977; 60: 810–14.
10. Weibel RE, Neff BJ, Kuter BJ, et al. Live attenuated varicella virus vaccine. efficacy trial in healthy children. N Engl J Med 1984; 310: 1409–15.
11. Monath TP, Cetron MS, McCarthy K, et al. Yellow fever 17D vaccine safety and immunogenicity in the elderly. Hum Vaccin 2005; 1: 207–14.
12. Monath TP, Nichols R, Archambault WT, et al. Comparative safety and immunogenicity of two yellow fever 17D vaccines (ARILVAX and YF-VAX) in a phase III multicenter, double-blind clinical trial. Am J Trop Med Hyg 2002; 66: 533–41.
13. Wahdan MH, Serie C, Cerisier Y, et al. A controlled field trial of live Salmonella typhi strain Ty 21a oral vaccine against typhoid: three-year results. J Infect Dis 1982; 145: 292–5.
14. Wahdan MH, Serie C, Germanier R, et al. A controlled field trial of liver oral typhoid vaccine Ty21a. Bull World Health Organ 1980; 58: 469–74.
15. Fogel A, Moshkowitz A, Rannon L, et al. Comparative trials of RA 27-3 and Cendehill rubella vaccines in adult and adolescent females. Am J Epidemiol 1971; 93: 392–8.
16. Vesikari T, la-Laurila EL, Heikkinen A, et al. Clinical trial of a new trivalent measles-mumps-rubella vaccine in young children. Am J Dis Child 1984; 138: 843–7.
17. Vesikari T, Karvonen A, Korhonen T, et al. Safety and immunogenicity of RIX4414 live attenuated human rotavirus vaccine in adults, toddlers and previously uninfected infants. Vaccine 2004; 22: 2836–42.
18. Furesz J, Nagler FP. Vaccination of school children with live mumps virus vaccine. Can Med Assoc J 1970; 102: 1153–5.
19. Borgono JM, Greiber R, Concha F, et al. Evaluation of live virus strain L-16-SSW measles vaccine. Rev Med Chil 1972; 100: 1133–4.
20. Belshe R, Lee MS, Walker RE, et al. Safety, immunogenicity and efficacy of intranasal, live attenuated influenza vaccine. Expert Rev Vaccines 2004; 3: 643–54.
21. Belshe RB, Mendelman PM. Safety and efficacy of live attenuated, cold-adapted, influenza vaccine-trivalent. Immunol Allergy Clin North Am 2003; 23: 745–67.
22. Belshe RB, Nichol KL, Black SB, et al. Safety, efficacy, and effectiveness of live, attenuated, cold-adapted influenza vaccine in an indicated population aged 5-49 years. Clin Infect Dis 2004; 39: 920–7.
23. CDC. Tuberculosis (TB) Fact Sheet. United States Government: Dept of Health and Human Services: Centers for Disease Control and Prevention, 2011. [Available online at http://www.cdc.gov/tb/publications/factsheets/prevention/BCG.htm]
24. Kit S, Kit M. Genetically engineered herpesvirus vaccines. Accomplishments in pigs and prospects in humans. Prog Med Virol 1991; 38: 128–66.
25. Kit S. Genetically engineered vaccines for control of Aujeszky's disease (pseudorabies). Vaccine 1990; 8: 420–4.
26. Chatfield SN, Roberts M, Dougan G, et al. The development of oral vaccines against parasitic diseases utilizing live attenuated Salmonella. Parasitology 1995; 110(Suppl): S17–24.
27. Mettenleiter TC. New developments in the construction of safer and more versatile pseudorabies virus vaccines. Dev Biol Stand 1995; 84: 83–7.
28. Kimman TG, Gielkens AL, Glazenburg K, et al. Characterization of live pseudorabies virus vaccines. Dev Biol Stand 1995; 84: 89–96.
29. Peterson LJ, Benson WW, Graeber FO. Vaccination-induced poliomyelitis in Idaho; preliminary report of experience with Salk poliomyelitis vaccine. J Am Med Assoc 1955; 159: 241–4.
30. Nathanson N, Langmuir AD. The cutter incident. poliomyelitis following formalde. Am J Hyg 1963; 78: 16–28.
31. Kapikian AZ, Mitchell RH, Chanock RM, et al. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated

with an inactivated RS virus vaccine. Am J Epidemiol 1969; 89: 405–21.
32. Kim HW, Canchola JG, Brandt CD, et al. Respiratory syncytial virus disease in infants despite prior administration of antigenic inactivated vaccine. Am J Epidemiol 1969; 89: 422–34.
33. Murphy BR, Prince GA, Walsh EE, et al. Dissociation between serum neutralizing and glycoprotein antibody responses of infants and children who received inactivated respiratory syncytial virus vaccine. J Clin Microbiol 1986; 24: 197–202.
34. Connors M, Kulkarni AB, F-irestone CY, et al. Pulmonary histopathology induced by respiratory syncytial virus (RSV) challenge of formalin-inactivated RSV-immunized BALB/c mice is abrogated by depletion of CD4+ T cells. J Virol 1992; 66: 7444–51.
35. Campbell JD, Clement KH, Wasserman SS, et al. Safety, reactogenicity and immunogenicity of a recombinant protective antigen anthrax vaccine given to healthy adults. Hum Vaccin 2007; 3: 205–11.
36. Sanofi Pasteur. Polio Vaccine Inactived IPOL. United States Government Federal Drug Administration (FDA), 2011. [Available online at http://www.fda.gov/downloads/biologicsbloodvaccines/vaccines/approvedproducts/ucm133479.pdf]
37. Noskova AV, Gorbunov MA, Pavlova LI, et al. Comparative study of the reactivity, safety and immunogenicity of "Havrix" inactivated hepatitis A vaccine. Vopr Virusol 2000; 45: 42–4.
38. Werzberger A, Mensch B, Kuter B, et al. A controlled trial of a formalin-inactivated hepatitis a vaccine in healthy children. N Engl J Med 1992; 327: 453–7.
39. Bernard KW, Roberts MA, Sumner J, et al. Human diploid cell rabies vaccine. effectiveness of immunization with small intradermal or subcutaneous doses. JAMA 1982; 247: 1138–42.
40. CDC. MMWR: Notice to Readers Availability of New Rabies Vaccine for Human Use. United States Government: Dept of Health and Human Services: Centers for Disease Control and Prevention, 2011. [Available online at http://www.cdc.gov/mmwr/ preview/mmwrhtml/00050848.htm]
41. Duggan ST, Plosker GL. Japanese encephalitis vaccine (inactivated, adsorbed) [IXIARO]. Drugs 2009; 69: 115–22.
42. Monath TP, Guirakhoo F, Nichols R, et al. Chimeric live, attenuated vaccine against Japanese encephalitis (ChimeriVax-JE): phase 2 clinical trials for safety and immunogenicity, effect of vaccine dose and schedule, and memory response to challenge with inactivated Japanese encephalitis antigen. J Infect Dis 2003; 188: 1213–30.
43. CDC. Vaccines and Preventable Diseases:Seasonal Influenza (Flu) Vaccination. United States Government: Dept of Health and Human Services: Centers for Disease Control and Prevention, 2011. [Available online at http://cdc.gov/Vaccines/vpd-vac/flu/default.htm]
44. CDC. Tetanus (Lockjaw) Vaccination. United States Government: Dept of Health and Human Services: Centers for Disease Control and Prevention, 2011. [Available online at http://www.cdc.gov/vaccines/vpd-vac/tetanus/default.htm]
45. Van DP, Burgess M. Immunogenicity of a combined diphtheria-tetanus-acellular pertussis vaccine in adults. Vaccine 2004; 22: 305–8.
46. Briand JP, Benkirane N, Guichard G, et al. A retro-inverso peptide corresponding to the GH loop of foot-and-mouth disease virus elicits high levels of long-lasting protective neutralizing antibodies. Proc Natl Acad Sci U.S.A 1997; 94: 12545–50.
47. Brown F. Foot-and-mouth disease and beyond: vaccine design, past, present and future. Arch Virol Suppl 1999; 15: 179–88.
48. Marciani DJ, Kensil CR, Beltz GA, et al. Genetically-engineered subunit vaccine against feline leukaemia virus: protective immune response in cats. Vaccine 1991; 9: 89–96.
49. Valenzuela P, Medina A, Rutter WJ, et al. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. Nature 1982; 298: 347–50.
50. Brideau RJ, Walters RR, Stier MA, et al. Protection of cotton rats against human respiratory syncytial virus by vaccination with a novel chimeric FG glycoprotein. J Gen Virol 1989; 70: 2637–44.
51. Brideau RJ, Oien NL, Lehman DJ, et al. Protection of cotton rats against human parainfluenza virus type 3 by vaccination with a chimeric FHN subunit glycoprotein. J Gen Virol 1993; 74: 471–7.
52. Kowalski J, Gilbert SA, van Drunen-Littel-van den Hurk S, et al. Heat-shock promoter-driven synthesis of secreted bovine herpesvirus glycoproteins in transfected cells. Vaccine 1993; 11: 1100–7.
53. Treanor JJ, Betts RF, Smith GE, et al. Evaluation of a recombinant hemagglutinin expressed in insect cells as an influenza vaccine in young and elderly adults. J Infect Dis 1996; 173: 1467–70.
54. Lakey DL, Treanor JJ, Betts RF, et al. Recombinant baculovirus influenza A hemagglutinin vaccines are well tolerated and immunogenic in healthy adults. J Infect Dis 1996; 174: 838–41.
55. Treanor JJ, Schiff GM, Hayden FG, et al. Safety and immunogenicity of a baculovirus-expressed hemagglutinin influenza vaccine: a randomized controlled trial. JAMA 2007; 297: 1577–82.
56. Crovari P, Crovari PC, Petrilli RC, et al. Immunogenicity of a yeast-derived hepatitis B vaccine (Engerix-B) in healthy young adults. Postgrad Med J 1987; 63: 161–4.
57. Diaz-Arnold AM, Leary JM. Vaccination against hepatitis B: the Heptavax-B and Recombivax-HB vaccines. Dentistry 1989; 9: 21–4.
58. Block SL, Brown DR, Chatterjee A, et al. Clinical trial and post-licensure safety profile of a prophylactic human papillomavirus (types 6, 11, 16, and 18) l1 virus-like particle vaccine. Pediatr Infect Dis J 2010; 29: 95–101.
59. Block SL, Nolan T, Sattler C, et al. Comparison of the immunogenicity and reactogenicity of a prophylactic quadrivalent human papillomavirus (types 6, 11, 16, and 18) L1 virus-like particle vaccine in male and female adolescents and young adult women. Pediatrics 2006; 118: 2135–45.
60. Szarewski A, Poppe W, Skinner SR, et al. Efficacy of the human papillomavirus (HPV)-16/18 AS04-adjuvanted vaccine in women aged 15-25 years with and without serological evidence of previous exposure to HPV-16/18. Int J Cancer 2012; 131: 106–16.
61. Deeks ED. Meningococcal quadrivalent (serogroups A, C, w135, and y) conjugate vaccine (Menveo): in adolescents and adults. BioDrugs 2010; 24: 287–97.
62. Keyserling H, Papa T, Koranyi K, et al. Safety, immunogenicity, and immune memory of a novel meningococcal (groups A, C, Y, and W-135) polysaccharide diphtheria toxoid conjugate vaccine (MCV-4) in healthy adolescents. Arch Pediatr Adolesc Med 2005; 159: 907–13.
63. Anderson EL, Kennedy DJ, Geldmacher KM, et al. Immunogenicity of heptavalent pneumococcal conjugate vaccine in infants. J Pediatr 1996; 128: 649–53.
64. Esposito S, Tansey S, Thompson A, et al. Safety and immunogenicity of a 13-valent pneumococcal conjugate vaccine compared to those of a 7-valent pneumococcal conjugate vaccine given as a three-dose series with routine vaccines in healthy infants and toddlers. Clin Vaccine Immunol 2010; 17: 1017–26.
65. Tartaglia J, Jarrett O, Neil JC, et al. Protection of cats against feline leukemia virus by vaccination with a canarypox virus recombinant, ALVAC-FL. J Virol 1993; 67: 2370–5.
66. Poulet H, Brunet S, Boularand C, et al. Efficacy of a canarypox virus-vectored vaccine against feline leukaemia. Vet Rec 2003; 153: 141–5.

67. El GH, Minke JM, Rehder J, et al. A West Nile virus (WNV) recombinant canarypox virus vaccine elicits WNV-specific neutralizing antibodies and cell-mediated immune responses in the horse. Vet Immunol Immunopathol 2008; 123: 230–9.
68. Minke JM, Siger L, Karaca K, et al. Recombinant canarypox-virus vaccine carrying the prM/E genes of West Nile virus protects horses against a West Nile virus-mosquito challenge. Arch Virol Suppl 2004; 221–30.
69. Minke JM, Toulemonde CE, Dinic S, et al. Effective priming of foals born to immune dams against influenza by a canarypox-vectored recombinant influenza H3N8 vaccine. J Comp Pathol 2007; 137:S76–80.
70. Minke JM, Toulemonde CE, Coupier H, et al. Efficacy of a canarypox-vectored recombinant vaccine expressing the hemagglutinin gene of equine influenza H3N8 virus in the protection of ponies from viral challenge. Am J Vet Res 2007; 68: 213–19.
71. Taylor J, Tartaglia J, Riviere M, et al. Applications of canarypox (ALVAC) vectors in human and veterinary vaccination. Dev Biol Stand 1994; 82: 131–5.
72. Taylor J, Trimarchi C, Weinberg R, et al. Efficacy studies on a canarypox-rabies recombinant virus. Vaccine 1991; 9: 190–3.
73. Cadoz M, Strady A, Meignier B, et al. Immunisation with canarypox virus expressing rabies glycoprotein. Lancet 1992; 339: 1429–32.
74. Pardo MC, Bauman JE, Mackowiak M. Protection of dogs against canine distemper by vaccination with a canarypox virus recombinant expressing canine distemper virus fusion and hemagglutinin glycoproteins. Am J Vet Res 1997; 58: 833–6.
75. Kyriakis CS, De VA, Barbe F, et al. Safety, immunogenicity and efficacy of poxvirus-based vector vaccines expressing the haemagglutinin gene of a highly pathogenic H5N1 avian influenza virus in pigs. Vaccine 2009; 27: 2258–64.
76. Boursnell ME, Green PF, Samson AC, et al. A recombinant fowlpox virus expressing the hemagglutinin-neuraminidase gene of Newcastle disease virus (NDV) protects chickens against challenge by NDV. Virology 1990; 178: 297–300.
77. Hanlon CL, Hayes DE, Hamir AN, et al. Proposed field evaluation of a rabies recombinant vaccine for raccoons (Procyon lotor): site selection, target species characteristics, and placebo baiting trials. J Wildl Dis 1989; 25: 555–67.
78. Romer-Oberdorfer A, Veits J, Helferich D, et al. Level of protection of chickens against highly pathogenic H5 avian influenza virus with Newcastle disease virus based live attenuated vector vaccine depends on homology of H5 sequence between vaccine and challenge virus. Vaccine 2008; 26: 2307–13.
79. Le Gros FX, Dancer A, Giacomini C, et al. Field efficacy trial of a novel HVT-IBD vector vaccine for 1-day-old broilers. Vaccine 2009; 27: 592–6.
80. Ma JK, Vine ND. Plant expression systems for the production of vaccines. Curr Top Microbiol Immunol 1999; 236: 275–92.
81. Ma JK, Hiatt A, Hein M, et al. Generation and assembly of secretory antibodies in plants. Science 1995; 268: 716–19.
82. Haq TA, Mason HS, Clements JD, et al. Oral immunization with a recombinant bacterial antigen produced in transgenic plants. Science 1995; 268: 714–16.
83. Carrillo C, Wigdorovitz A, Oliveros JC, et al. Protective immune response to foot-and-mouth disease virus with VP1 expressed in transgenic plants. J Virol 1998; 72: 1688–90.
84. Dalsgaard K, Uttenthal A, Jones TD, et al. Plant-derived vaccine protects target animals against a viral disease. Nat Biotechnol 1997; 15: 248–52.
85. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. J Immunol Methods 1994; 171: 131–7.
86. Arntzen CJ, Mason HS, Shi J, et al. Production of candidate oral vaccines in edible tissues of transgenic plants. In: Brown F, Chanock RM, Ginsberg HS, Lerner RA, eds. Vaccines '94: Modern Approaches to New Vaccines Including Prevention of AIDS. New York: Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1994: 339–44.
87. Zanetti SE, Salk J. The immunology of new generation vaccines. Immunol Today 1987; 8: 18–22.
88. Oosterlaken TA, Harmsen M, Jhagjhoor-Singh SS, et al. A protective monoclonal anti-idiotypic vaccine to lethal Semliki Forest virus infection in BALB/c mice. J Virol 1991; 65: 98–102.
89. Roy P, Noad R. Virus-like particles as a vaccine delivery system: myths and facts. Adv Exp Med Biol 2009; 655: 145–58.
90. Jennings GT, Bachmann MF. Immunodrugs: therapeutic VLP-based vaccines for chronic diseases. Annu Rev Pharmacol Toxicol 2009; 49: 303–26.
91. Chackerian B. Virus-like particles: flexible platforms for vaccine development. Expert Rev Vaccines 2007; 6: 381–90.
92. Hunter Z, Smyth HD, Durfee P, et al. Induction of mucosal and systemic antibody responses against the HIV coreceptor CCR5 upon intramuscular immunization and aerosol delivery of a virus-like particle based vaccine. Vaccine 2009; 28: 403–14.
93. Barassi C, Soprana E, Pastori C, et al. Induction of murine mucosal CCR5-reactive antibodies as an anti-human immunodeficiency virus strategy. J Virol 2005; 79: 6848–58.
94. Mastico RA, Talbot SJ, Stockley PG. Multiple presentation of foreign peptides on the surface of an RNA-free spherical bacteriophage capsid. J Gen Virol 1993; 74: 541–8.
95. Liu WJ, Liu XS, Zhao KN, et al. Papillomavirus virus-like particles for the delivery of multiple cytotoxic T cell epitopes. Virology 2000; 273: 374–82.
96. Maurer P, Jennings GT, Willers J, et al. A therapeutic vaccine for nicotine dependence: preclinical efficacy, and Phase I safety and immunogenicity. Eur J Immunol 2005; 35: 2031–40.
97. Wolff JA, Malone RW, Williams P, et al. Direct gene transfer into mouse muscle in vivo. Science 1990; 247: 1465–8.
98. Yang NS, Burkholder J, Roberts B, et al. In vivo and in vitro gene transfer to mammalian somatic cells by particle bombardment. Proc Natl Acad Sci USA 1990; 87: 9568–72.
99. Williams RS, Johnston SA, Riedy M, et al. Introduction of foreign genes into tissues of living mice by DNA-coated microprojectiles. Proc Natl Acad Sci USA 1991; 88: 2726–30.
100. Felgner PL, Ringold GM. Cationic liposome-mediated transfection. Nature 1989; 337: 387–8.
101. Barthel F, Remy JS, Loeffler JP, et al. Gene transfer optimization with lipospermine-coated DNA. DNA Cell Biol 1993; 12: 553–60.
102. Mathiowitz E, Jacob JS, Jong YS, et al. Biologically erodable microspheres as potential oral drug delivery systems. Nature 1997; 386: 410–14.
103. Zhao K, Shi X, Zhao Y, et al. Preparation and immunological effectiveness of a swine influenza DNA vaccine encapsulated in chitosan nanoparticles. Vaccine 2011; 29: 8549–56.
104. Huang JL, Yin YX, Pan ZM, et al. Intranasal immunization with chitosan/pCAGGS-flaA nanoparticles inhibits Campylobacter jejuni in a White Leghorn model. J Biomed Biotechnol 2010; 2010: 1–8.
105. Lee PW, Hsu SH, Tsai JS, et al. Multifunctional core-shell polymeric nanoparticles for transdermal DNA delivery and epidermal Langerhans cells tracking. Biomaterials 2010; 31: 2425–34.
106. Hallengard D, Haller BK, Maltais AK, et al. Comparison of plasmid vaccine immunization schedules using intradermal in vivo electroporation. Clin Vaccine Immunol 2011; 18: 1577–81.
107. Dupuy LC, Richards MJ, Ellefsen B, et al. A DNA vaccine for venezuelan equine encephalitis virus delivered by intramuscular electroporation elicits high levels of neutralizing antibodies in multiple animal models and provides protective immunity to mice and nonhuman primates. Clin Vaccine Immunol 2011; 18: 707–16.

108. Lin F, Shen X, McCoy JR, et al. A novel prototype device for electroporation-enhanced DNA vaccine delivery simultaneously to both skin and muscle. Vaccine 2011; 29: 6771–80.
109. Aggarwal N, HogenEsch H, Guo P, et al. Biodegradable alginate microspheres as a delivery system for naked DNA. Can J Vet Res 1999; 63: 148–52.
110. Mittal SK, Aggarwal N, Sailaja G, et al. Immunization with DNA, adenovirus or both in biodegradable alginate microspheres: effect of route of inoculation on immune response. Vaccine 2000; 19: 253–63.
111. Ulmer JB, Donnelly JJ, Parker SE, et al. Heterologous protection against influenza by injection of DNA encoding a viral protein. Science 1993; 259: 1745–9.
112. Donnelly JJ, Ulmer JB, Liu MA. DNA vaccines. Life Sci 1997; 60: 163–72.
113. Lowrie DB, Silva CL, Colston MJ, et al. Protection against tuberculosis by a plasmid DNA vaccine. Vaccine 1997; 15: 834–8.
114. Lowrie DB, Tascon RE, Bonato VL, et al. Therapy of tuberculosis in mice by DNA vaccination. Nature 1999; 400: 269–71.
115. Koprowski H, Weiner DB. DNA vaccination/genetic vaccination. Curr Top Microbiol Immunol 1998; 226: V–XIII.
116. Morrow CD, Novak MJ, Ansardi DC, et al. Recombinant viruses as vectors for mucosal immunity. Curr Top Microbiol Immunol 1999; 236: 255–73.
117. Sharma A, Tandon M, Bangari DS, et al. Adenoviral vector-based strategies for cancer therapy. Curr Drug ther 2009; 4: 117–38.
118. Sharma A, Li X, Bangari DS, et al. Adenovirus receptors and their implications in gene delivery. Virus Res 2009; 143: 184–94.
119. Tandon M, Vemula SV, Mittal SK. Emerging strategies for EphA2 receptor targeting for cancer. Expert Opin Ther Targets 2011; 15: 31–51.
120. Bangari DS, Mittal SK. Development of nonhuman adenoviruses as vaccine vectors. Vaccine 2006; 24: 849–62.
121. Rouse BT, Sehrawat S. Immunity and immunopathology to viruses: what decides the outcome? Nat Rev Immunol 2010; 10: 514–26.
122. Vemula SV, Mittal SK. Production of adenovirus vectors and their use as a delivery system for influenza vaccines. Expert Opin Biol Ther 2010; 10: 1469–87.
123. Hoelscher MA, Garg S, Bangari DS, et al. Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. Lancet 2006; 367: 475–81.
124. Breker-Klassen MM, Yoo D, Mittal SK, et al. Recombinant type 5 adenoviruses expressing bovine parainfluenza virus type 3 glycoproteins protect Sigmodon hispidus cotton rats from bovine parainfluenza virus type 3 infection. J Virol 1995; 69: 4308–15.
125. Singh N, Pandey A, Jayashankar L, et al. Bovine adenoviral vector-based H5N1 influenza vaccine overcomes exceptionally high levels of pre-existing immunity against human adenovirus. Mol Ther 2008; 16: 965–71.
126. Muster T, Ferko B, Klima A, et al. Mucosal model of immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. J Virol 1995; 69: 6678–86.
127. Lawrence DN, Goldenthal KL, Boslego JW, et al. Public health implications of emerging vaccine technologies. Pharm Biotechnol 1995; 6: 43–60.
128. Pastoret PP, Brochier B, Languet B, et al. First field trial of fox vaccination against rabies using a vaccinia-rabies recombinant virus. Vet Rec 1988; 123: 481–3.
129. Robbins AH, Borden MD, Windmiller BS, et al. Prevention of the spread of rabies to wildlife by oral vaccination of raccoons in Massachusetts. J Am Vet Med Assoc 1998; 213: 1407–12.
130. Taylor J, Paoletti E. Pox viruses as eukaryotic cloning and expression vectors: future medical and veterinary vaccines. Prog Vet Microbiol Immunol 1988; 4: 197–217.
131. Graham FL, Prevec L. Adenovirus-based expression vectors and recombinant vaccines. Biotechnology 1992; 20: 363–90.
132. Imler JL. Adenovirus vectors as recombinant viral vaccines. Vaccine 1995; 13: 1143–51.
133. Mittal SK, Papp Z, Tikoo SK, et al. Induction of systemic and mucosal immune responses in cotton rats immunized with human adenovirus type 5 recombinants expressing the full and truncated forms of bovine herpesvirus type 1 glycoprotein gD. Virology 1996; 222: 299–309.
134. Davis BS, Chang GJ, Cropp B, et al. West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays. J Virol 2001; 75: 4040–7.
135. Purcell MK, Nichols KM, Winton JR, et al. Comprehensive gene expression profiling following DNA vaccination of rainbow trout against infectious hematopoietic necrosis virus. Mol Immunol 2006; 43: 2089–106.
136. Bergman PJ, Camps-Palau MA, McKnight JA, et al. Development of a xenogeneic DNA vaccine program for canine malignant melanoma at the animal medical center. Vaccine 2006; 24: 4582–5.
137. Bergman PJ, McKnight J, Novosad A, et al. Long-term survival of dogs with advanced malignant melanoma after DNA vaccination with xenogeneic human tyrosinase: a phase I trial. Clin Cancer Res 2003; 9: 1284–90.
138. Jacobs WR Jr, Snapper SB, Lugosi L, et al. Development of BCG as a recombinant vaccine vehicle. Curr Top Microbiol Immunol 1990; 155: 153–60.
139. Arenas-Gamboa AM, Ficht TA, Davis DS, et al. Oral vaccination with microencapsuled strain 19 vaccine confers enhanced protection against Brucella abortus strain 2308 challenge in red deer (Cervus elaphus elaphus). J Wild Dis 2009; 45: 1021–9.
140. Ryan ET, Crean TI, John M, et al. In vivo expression and immunoadjuvancy of a mutant of heat-labile enterotoxin of Escherichia coli in vaccine and vector strains of Vibrio cholerae. Infect Immun 1999; 67: 1694–701.
141. Cardenas L, Dasgupta U, Clements JD. Influence of strain viability and antigen dose on the use of attenuated mutants of Salmonella as vaccine carriers. Vaccine 1994; 12: 833–40.
142. Cardenas L, Clements JD. Oral immunization using live attenuated Salmonella spp. as carriers of foreign antigens. Clin Microbiol Rev 1992; 5: 328–42.
143. Killeen K, Spriggs D, Mekalanos J. Bacterial mucosal vaccines: Vibrio cholerae as a live attenuated vaccine/vector paradigm. Curr Top Microbiol Immunol 1999; 236: 237–54.
144. Nakayama K, Kelly SM, Curtiss R. Construction of an ASD+ expression-cloning vector: stable maintenance and high level expression of cloned genes in a salmonella vaccine strain. Biotechnology 1998; 6: 693–7.
145. Gentschev I, Hess J, Goebel W. Change in the cellular localization of alkaline phosphatase by alteration of its carboxy-terminal sequence. Mol Gen Genet 1990; 222: 211–16.
146. Tzschaschel BD, Klee SR, de LV, et al. Towards a vaccine candidate against Shigella dysenteriae 1: expression of the Shiga toxin B-subunit in an attenuated Shigella flexneri aroD carrier strain. Microb Pathog 1996; 21: 277–88.
147. Kalinski P, Hilkens CM, Wierenga EA, et al. T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal. Immunol Today 1999; 20: 561–7.
148. Rock KL, Latz E, Ontiveros F, et al. The sterile inflammatory response. Annu Rev Immunol 2010; 28: 321–42.
149. Hem SL, HogenEsch H. Relationship between physical and chemical properties of aluminum-containing adjuvants and immunopotentiation. Expert Rev Vaccines 2007; 6: 685–98.
150. Iyer S, HogenEsch H, Hem SL. Effect of the degree of phosphate substitution in aluminum hydroxide adjuvant on the adsorption of phosphorylated proteins. Pharm Dev Technol 2003; 8: 81–6.

151. Kwissa M, Lindblad EB, Schirmbeck R, et al. Codelivery of a DNA vaccine and a protein vaccine with aluminum phosphate stimulates a potent and multivalent immune response. J Mol Med (Berl) 2003; 81: 502–10.
152. Mohr E, Cunningham AF, Toellner KM, et al. IFN-gamma produced by CD8 T cells induces T-bet-dependent and -independent class switching in B cells in responses to alum-precipitated protein vaccine. Proc Natl Acad Sci USA 2010; 107: 17292–7.
153. MacLeod MK, McKee AS, David A, et al. Vaccine adjuvants aluminum and monophosphoryl lipid A provide distinct signals to generate protective cytotoxic memory CD8 T cells. Proc Natl Acad Sci USA 2011; 108: 7914–19.
154. Glenny AT, Buttle AH, Stevens MF. Rate of disappearance of diphtheria toxoid injected into rabbits and guinea - pigs: Toxoid precipitated with alum. J Pathol Bacteriol 1931; 34: 267–75.
155. Romero Mendez IZ, Shi Y, HogenEsch H, et al. Potentiation of the immune response to non-adsorbed antigens by aluminum-containing adjuvants. Vaccine 2007; 25: 825–33.
156. Noe SM, Green MA, HogenEsch H, et al. Mechanism of immunopotentiation by aluminum-containing adjuvants elucidated by the relationship between antigen retention at the inoculation site and the immune response. Vaccine 2010; 28: 3588–94.
157. Goto N, Akama K. Histopathological studies of reactions in mice injected with aluminum-adsorbed tetanus toxoid. Microbiol Immunol 1982; 26: 1121–32.
158. Kool M, Soullie T, van NM, et al. Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J Exp Med 2008; 205: 869–82.
159. Sokolovska A, Hem SL, HogenEsch H. Activation of dendritic cells and induction of CD4(+) T cell differentiation by aluminum-containing adjuvants. Vaccine 2007; 25: 4575–85.
160. Li H, Nookala S, Re F. Aluminum hydroxide adjuvants activate caspase-1 and induce IL-1beta and IL-18 release. J Immunol 2007; 178: 5271–6.
161. Eisenbarth SC, Colegio OR, O'Connor W, et al. Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants. Nature 2008; 453: 1122–6.
162. Li H, Willingham SB, Ting JP, et al. Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. J Immunol 2008; 181: 17–21.
163. Franchi L, Nunez G. The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1beta secretion but dispensable for adjuvant activity. Eur J Immunol 2008; 38: 2085–9.
164. Ng G, Sharma K, Ward SM, et al. Receptor-independent, direct membrane binding leads to cell-surface lipid sorting and Syk kinase activation in dendritic cells. Immunity 2008; 29: 807–18.
165. Flach TL, Ng G, Hari A, et al. Alum interaction with dendritic cell membrane lipids is essential for its adjuvanticity. Nat Med 2011; 17: 479–87.
166. Garcon N, Van Mechelen M, Wettendorff M. Development and evaluation of AS04, a novel and improved adjuvant system containing MPL and aluminum salt. In: O'Hagan DT, ed. Immunopotentiators in Modern Vaccines. San Diego: Elsevier Academic Press, 2006: 161–77.
167. Mata-Haro V, Cekic C, Martin M, et al. The vaccine adjuvant monophosphoryl lipid a as a TRIF-biased agonist of TLR4. Science 2007; 316: 1628–32.
168. Didierlaurent AM, Morel S, Lockman L, et al. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. J Immunol 2009; 183: 6186–97.
169. O'Hagan DT, Rappuoli R, De GE, et al. MF59 adjuvant: the best insurance against influenza strain diversity. Expert Rev Vaccines 2011; 10: 447–62.
170. Ellebedy AH, Lupfer C, Ghoneim HE, et al. Inflammasome-independent role of the apoptosis-associated speck-like protein containing CARD (ASC) in the adjuvant effect of MF59. Proc Natl Acad Sci USA 2011; 108: 2927–32.
171. Mosca F, Tritto E, Muzzi A, et al. Molecular and cellular signatures of human vaccine adjuvants. Proc Natl Acad Sci USA 2008; 105: 10501–6.
172. Calabro S, Tortoli M, Baudner BC, et al. Vaccine adjuvants alum and MF59 induce rapid recruitment of neutrophils and monocytes that participate in antigen transport to draining lymph nodes. Vaccine 2011; 29: 1812–23.
173. Sun HX, Xie Y, Ye YP. ISCOMs and ISCOMATRIX. Vaccine 2009; 27: 4388–401.
174. Brokstad KA, Cox RJ, Olofsson J, et al. Parenteral influenza vaccination induces a rapid systemic and local immune response. J Infect Dis 1995; 171: 198–203.
175. Shroff KE, Marcucci-Borges LA, de Bruin SJ, et al. Induction of HSV-gD2 specific CD4(+) cells in Peyer's patches and mucosal antibody responses in mice following DNA immunization by both parenteral and mucosal administration. Vaccine 1999; 18: 222–30.
176. Lundholm P, Asakura Y, Hinkula J, et al. Induction of mucosal IgA by a novel jet delivery technique for HIV-1 DNA. Vaccine 1999; 17: 2036–42.
177. McCluskie MJ, Brazolot Millan CL, Gramzinski RA, et al. Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates. Mol Med 1999; 5: 287–300.
178. Langue J, Ethevenaux C, Champsaur A, et al. Safety and immunogenicity of Haemophilus influenzae type b-tetanus toxoid conjugate, presented in a dual-chamber syringe with diphtheria-tetanus-pertussis and inactivated poliomyelitis combination vaccine. Eur J Pediatr 1999; 158: 717–22.
179. Kanra G, Yurdakok K, Ceyhan M, et al. Immunogenicity and safety of Haemophilus influenzae type b capsular polysaccharide tetanus conjugate vaccine (PRP-T) presented in a dual-chamber syringe with DTP. Acta Paediatr Jpn 1997; 39: 676–80.
180. Shi Z, Curiel DT, Tang DC. DNA-based non-invasive vaccination onto the skin. Vaccine 1999; 17: 2136–41.
181. Misra A, Ganga S, Upadhyay P. Needle-free, non-adjuvanted skin immunization by electroporation-enhanced transdermal delivery of diphtheria toxoid and a candidate peptide vaccine against hepatitis B virus. Vaccine 1999; 18: 517–23.
182. Glenn GM, Scharton-Kersten T, Vassell R, et al. Transcutaneous immunization with bacterial ADP-ribosylating exotoxins as antigens and adjuvants. Infect Immun 1999; 67: 1100–6.
183. Scharton-Kersten T, Glenn GM, Vassell R, et al. Principles of transcutaneous immunization using cholera toxin as an adjuvant. Vaccine 1999; 1:S37–43.
184. Glenn GM, Scharton-Kersten T, Vassell R, et al. Transcutaneous immunization with cholera toxin protects mice against lethal mucosal toxin challenge. J Immunol 1998; 161: 3211–14.
185. Arora A, Prausnitz MR, Mitragotri S. Micro-scale devices for transdermal drug delivery. Int J Pharm 2008; 364: 227–36.
186. Prausnitz MR, Langer R. Transdermal drug delivery. Nat Biotechnol 2008; 26: 1261–8.
187. Sivamani RK, Stoeber B, Liepmann D, et al. Microneedle penetration and injection past the stratum corneum in humans. J Dermatolog Treat 2009; 20: 156–9.
188. McGhee JR, Mestecky J, Dertzbaugh MT, et al. The mucosal immune system: from fundamental concepts to vaccine development. Vaccine 1992; 10: 75–88.
189. Hornquist E, Lycke N, Czerkinsky C, et al. Cholera toxin and cholera B subunit as oral-mucosal adjuvant and antigen carrier systems. In: O'Hagan D, ed. Novel Delivery Systems for Oral Vaccines. Ann Arbor MI: CRC Press, Inc, 1994: 157–73.
190. Service RF. Triggering the first line of defense. Science 1994; 265: 1522–4.

191. Heritage PL, Underdown BJ, Brook MA, et al. Oral administration of polymer-grafted starch microparticles activates gut-associated lymphocytes and primes mice for a subsequent systemic antigen challenge. Vaccine 1998; 16: 2010–17.
192. Dale JW, Dellagostin OA, Norman E, et al. Multivalent BCG vaccines. In: O'Hagan D, ed. Novel Delivery Systems for Oral Vaccines. Ann Arbor MI: CRC Press, Inc, 1994: 87–109.
193. Husby S, Jensenius JC, Svehag SE. Passage of undegraded dietary antigen into the blood of healthy adults. further characterization of the kinetics of uptake and the size distribution of the antigen. Scand J Immunol 1986; 24: 447–55.
194. Eaton KA, Krakowka S. Chronic active gastritis due to Helicobacter pylori in immunized gnotobiotic piglets. Gastroenterology 1992; 103: 1580–6.
195. O'Hagan D. Microparticles as oral vaccines. In: Novel Delivery Systems for Oral Vaccines. Ann Arbor MI: CRC Press, Inc, 1994: 175–205.
196. van der Lubben IM, van Opdorp FA, Hengeveld MR, et al. Transport of chitosan microparticles for mucosal vaccine delivery in a human intestinal M-cell model. J Drug Target 2002; 10: 449–56.
197. Stertman L, Lundgren E, Sjoholm I. Starch microparticles as a vaccine adjuvant: only uptake in Peyer's patches decides the profile of the immune response. Vaccine 2006; 24: 3661–8.
198. Foster N, Clark MA, Jepson MA, et al. Ulex europaeus 1 lectin targets microspheres to mouse Peyer's patch M-cells in vivo. Vaccine 1998; 16: 536–41.
199. Ball JM, Graham DY, Opekun AR, et al. Recombinant Norwalk virus-like particles given orally to volunteers: phase I study. Gastroenterology 1999; 117: 40–8.
200. Eyles JE, Williamson ED, Alpar HO. Immunological responses to nasal delivery of free and encapsulated tetanus toxoid: studies on the effect of vehicle volume. Int J Pharm 1999; 189: 75–9.
201. Rudin A, Riise GC, Holmgren J. Antibody responses in the lower respiratory tract and male urogenital tract in humans after nasal and oral vaccination with cholera toxin B subunit. Infect Immun 1999; 67: 2884–90.
202. Hagenaars N, Mania M, de JP, et al. Role of trimethylated chitosan (TMC) in nasal residence time, local distribution and toxicity of an intranasal influenza vaccine. J Control Release 2010; 144: 17–24.
203. Ali T, Scott N, Kallas W, et al. Detection of influenza antigen with rapid antibody-based tests after intranasal influenza vaccination (FluMist). Clin Infect Dis 2004; 38: 760–2.
204. Brown AR, George DW, Matteson DK. Vaccinator device for delivering propellant-driven aerosols of Streptococcus suis bacterin into the respiratory tracts of swine. Vaccine 1997; 15: 1165–73.
205. LiCalsi C, Christensen T, Bennett JV, et al. Dry powder inhalation as a potential delivery method for vaccines. Vaccine 1999; 17: 1796–803.
206. McCluskie MJ, Chu Y, Xia JL, et al. Direct gene transfer to the respiratory tract of mice with pure plasmid and lipid-formulated DNA. Antisense Nucleic Acid Drug Dev 1998; 8: 401–14.
207. Weng CN, Tzan YL, Liu SD, et al. Protective effects of an oral microencapsulated Mycoplasma hyopneumoniae vaccine against experimental infection in pigs. Res Vet Sci 1992; 53: 42–6.
208. Morris W, Steinhoff MC, Russell PK. Potential of polymer microencapsulation technology for vaccine innovation. Vaccine 1994; 12: 5–11.
209. O'Hagan DT. Vaccine adjuvants. In: Preparation and Research Protocols. Totowa NJ: Humana Press, 2000: 342.
210. Kuntz RM, Saltzman WM. Polymeric controlled delivery for immunization. Trends Biotechnol 1997; 15: 364–9.
211. Suckow MA, Jarvinen LZ, HogenEsch H, et al. Immunization of rabbits against a bacterial pathogen with an alginate microparticle vaccine. J.Control Release 2002; 85: 227–35.
212. Desai MP, Labhasetwar V, Amidon GL, et al. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. Pharm Res 1996; 13: 1838–45.
213. Nakanishi T, Kunisawa J, Hayashi A, et al. Positively charged liposome functions as an efficient immunoadjuvant in inducing cell-mediated immune response to soluble proteins. J Control Release 1999; 61: 233–40.
214. Hayashi A, Nakanishi T, Kunisawa J, et al. A novel vaccine delivery system using immunopotentiating fusogenic liposomes. Biochem Biophys Res Commun 1999; 261: 824–8.
215. Lasic DD. <[13] Book Title>. Boca Raton FL: CRC Press, 1997: 67–112.
216. Poltl-Frank F, Zurbriggen R, Helg A, et al. Use of reconstituted influenza virus virosomes as an immunopotentiating delivery system for a peptide-based vaccine. Clin Exp Immunol 1999; 117: 496–503.
217. Cryz SJ. BERNA: a century of immunobiological innovation. Vaccine 1999; 17:S1–5.
218. Casal JI, Rueda P, Hurtado A. Parvovirus-like particles as vaccine vectors. Methods 1999; 19: 174–86.
219. Liu XS, Abdul-Jabbar I, Qi YM, et al. Mucosal immunisation with papillomavirus virus-like particles elicits systemic and mucosal immunity in mice. Virology 1998; 252: 39–45.
220. Rentel CO, Bouwstra JA, Naisbett B, et al. Niosomes as a novel peroral vaccine delivery system. Int J Pharm 1999; 186: 161–7.
221. St CN, Shenoy B, Jacob LD, et al. Cross-linked protein crystals for vaccine delivery. Proc Natl Acad Sci USA 1999; 96: 9469–74.
222. Park K. Nano is better than micro for targeted vaccine delivery. J Control Release 2010; 144: 117–17.
223. Cruz LJ, Tacken PJ, Fokkink R, et al. Targeted PLGA nano- but not microparticles specifically deliver antigen to human dendritic cells via DC-SIGN in vitro. J Control Release 2010; 144: 118–26.
224. Thompson CB, Allison JP. The emerging role of CTLA-4 as an immune attenuator. Immunity 1997; 7: 445–50.
225. Abrams JR, Lebwohl MG, Guzzo CA, et al. CTLA4Ig-mediated blockade of T-cell costimulation in patients with psoriasis vulgaris. J Clin Invest 1999; 103: 1243–52.
226. Kwon ED, Foster BA, Hurwitz AA, et al. Elimination of residual metastatic prostate cancer after surgery and adjunctive cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) blockade immunotherapy. Proc Natl Acad Sci USA 1999; 96: 15074–9.
227. Krieg AM, Yi AK, Hartmann G. Mechanisms and therapeutic applications of immune stimulatory cpG DNA. Pharmacol Ther 1999; 84: 113–20.
228. Klinman DM. Adjuvant activity of CpG oligodeoxynucleotides. Int Rev Immunol 2006; 25: 135–54.
229. Hall GL, Compston A, Scolding NJ. Beta-interferon and multiple sclerosis. Trends Neurosci 1997; 20: 63–7.
230. Yong VW, Chabot S, Stuve O, et al. Interferon beta in the treatment of multiple sclerosis: mechanisms of action. Neurology 1998; 51: 682–9.
231. Moreland LW. Inhibitors of tumor necrosis factor for rheumatoid arthritis. J Rheumatol Suppl 1999; 57: 7–15.
232. Sandborn WJ, Hanauer SB. Antitumor necrosis factor therapy for inflammatory bowel disease: a review of agents, pharmacology, clinical results, and safety. Inflamm Bowel Dis 1999; 5: 119–33.

Author Query Form			
Title	Vaccines, Adjuvants, and Delivery Systems for Infectious Diseases		
Chapter No.	342		
Dear Author/Publisher During the preparation of your manuscript for typesetting some questions have arisen. These are listed below. Please check your typeset proof carefully and mark any corrections in the margin of the proof or compile them as a separate list.			
Query Ref.	**Page No.**	**Details Required**	**Author's/Publisher's Response**
AQ1	6	Please check and confirm whether the insertion of citation for Table 4 in the text is OK.	