Vaccines, Adjuvants, and Delivery Systems

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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, revovirus, 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 smallpox 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
Temperature-sensitive mutants
Cold-adapted mutants
Site-directed mutants
Gene-deleted mutants
Gene reassessment mutants
Naturally available mutants
Mutants generated by adaptation to
Unnatural host

Mammalian cells
Bacteria
Insect cells
Plant cells

Viral vectors
Bacterial vectors

Plasmid DNA containing one or
more genes and delivered by
various methods

One or more viral proteins
leading to virus-like particles
(VLP) without the viral genome

DNA

VLP

Subunit

Attenuated

Wildtype organisms

Attenuated organisms

Inactivated

Inactivated organisms

Naturally occurring
Recombinant protein
Immunogenic peptides
Anti-idiotypic antibodies

VLP

DNA

Vector

Viral vectors

Bacterial vectors

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, ade-
novirus, and influenza virus. Thus, these mutants can repli-
cate 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 ear-
lier 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 patho-
gens 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 com-
plete 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 thymi-
dine kinase gene (non-structural protein involved in viral DNA
replication) and the gC, gG, and gE genes (non-essential gly-
coproteins involved in virus assembly) (24,25). A gene-deleted
vaccine of pseudorabies virus has proven highly effective in
eradicating pseudorabies in the United States. It has been dem-
onstrated 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 tar-
geted 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 associ-
ated with live vaccines include: (i) low cost of production
due to fewer numbers of organisms in the vaccine formula-
tion; (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 vac-
cine 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

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

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,
Vaccines, Adjuvants, and Delivery Systems

in bacteria induce protective immunity, e.g., gp70 of feline for their antigenicity. Some viral glycoproteins expressed processes. Other immunogenic proteins, especially of viral because the bacterial system lacks many post-translational systems. A particular bacterial expression system, processing have been well worked out for bacterial expres-
sion systems. A bacterial expression system at a low cost because the scale-up and downstream production can be easily purified, inactivated, and form is sometimes cumbersome and expensive. However, 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 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

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.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Inactivated vaccines for use in humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target pathogen</td>
<td>Attenuation methods</td>
</tr>
<tr>
<td>Anthrax</td>
<td>Formalin</td>
</tr>
<tr>
<td>Polio virus</td>
<td>Heat and formalin</td>
</tr>
<tr>
<td>Hepatitis A virus</td>
<td>Formalin</td>
</tr>
<tr>
<td>Rabies virus</td>
<td>β-Propiolactone</td>
</tr>
<tr>
<td>Japanese encephalitis virus</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Influenza virus</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Clostridium tetani</td>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Corynebacterium diphtheriae, Clostridium tetani, Bordetella pertussis</td>
<td>Glutaraldehyde and formaldehyde</td>
</tr>
</tbody>
</table>

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<table>
<thead>
<tr>
<th>Table 3</th>
<th>Subunit and conjugate vaccines for use in humans</th>
</tr>
</thead>
<tbody>
<tr>
<td>Target pathogen</td>
<td>Target antigens</td>
</tr>
<tr>
<td>Hepatitis B virus</td>
<td>Hepatitis B surface antigen</td>
</tr>
<tr>
<td>Human papilloma virus</td>
<td>HPV types 6, 11, 16 and 18</td>
</tr>
<tr>
<td>Human papilloma virus</td>
<td>HPV types 16 and 18</td>
</tr>
<tr>
<td>Neisseria meningitidis</td>
<td>Meningococcal polysaccharides</td>
</tr>
<tr>
<td>Streptococcus pneumoniae</td>
<td>Capsular polysaccharides</td>
</tr>
</tbody>
</table>

Subunit Vaccine 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 pathogens 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-Idiotype 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-idiotype 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-idiotype 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
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), biologically erodible 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 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 4** DNA vaccines for veterinary use

<table>
<thead>
<tr>
<th>Target pathogen/cancer</th>
<th>Delivery vector</th>
<th>Target antigen</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>West Nile Virus</td>
<td>DNA</td>
<td>preM and Env</td>
<td>(134)</td>
</tr>
<tr>
<td>Infectious hematopoietic necrosis virus</td>
<td>DNA</td>
<td>Glycoprotein</td>
<td>(135)</td>
</tr>
<tr>
<td>Canine melanoma</td>
<td>DNA</td>
<td>Human tyrosinase</td>
<td>(136,137)</td>
</tr>
</tbody>
</table>

**Vected 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).
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).

### Table 5 Viral vectored vaccines for veterinary use

<table>
<thead>
<tr>
<th>Target pathogen</th>
<th>Delivery vector</th>
<th>Target antigen</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feline leukemia virus</td>
<td>Canarypox virus</td>
<td>Envelope protein (Env), group-specific protein (gag), polymerase (pol)</td>
<td>(65,66)</td>
</tr>
<tr>
<td>West Nile Virus</td>
<td>Canarypox virus</td>
<td>Protein M precursor (preM) and Env</td>
<td>(67,68)</td>
</tr>
<tr>
<td>Equine influenza virus</td>
<td>Canarypox virus</td>
<td>Hemagglutinin (HA)</td>
<td>(69,70)</td>
</tr>
<tr>
<td>Feline rabies virus</td>
<td>Canarypox virus</td>
<td>Glycoprotein G</td>
<td>(71–73)</td>
</tr>
<tr>
<td>Canine distemper virus</td>
<td>Canarypox Vector</td>
<td>HA and fusion antigen (FA)</td>
<td>(74)</td>
</tr>
<tr>
<td>Avian influenza (AI) virus</td>
<td>Fowlpox virus (FPV)</td>
<td>HA</td>
<td>(75)</td>
</tr>
<tr>
<td>Newcastle disease virus (NDV)</td>
<td>FPV</td>
<td>HA, neuraminidase, and FA</td>
<td>(76)</td>
</tr>
<tr>
<td>Rabies virus</td>
<td>Vaccinia virus</td>
<td>Glycoprotein G</td>
<td>(77)</td>
</tr>
<tr>
<td>AI and ND</td>
<td>NDV</td>
<td>HA</td>
<td>(78)</td>
</tr>
<tr>
<td>Infectious bursal disease virus &amp; Marek's disease virus</td>
<td>Turkey herpes virus</td>
<td>Viral protein 2</td>
<td>(79)</td>
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### 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 their 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 (HMGBl), 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 GlaxoSmithKline 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, intra-rectal, 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 Intrajet®. They all use high pressure released in a very short period to deliver drugs through the skin. A jet-immunization technique was used for introral administration of DNA in the cheek, resulting in high IgA mucosal responses (176). The introra oral 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 micronneedle 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,
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.

Figure 3  Mucosal immunization leading to dispersal of IgA antibodies to common mucosal immune system.
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 completely (206). Such a complex formulation 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, neo-somes, 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), polystyrene-starch, dextran, albumin, and alginate 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 phospholipids. 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 immuno-potentiating 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-4 Ig, 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.

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ARTICLES OF FURTHER INTEREST

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Adjuvant, p.
Pandemic, p.
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