# VACCINES AND OTHER IMMUNOLOGICAL PRODUCTS

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## VACCINES

### Introduction

The concept of vaccination was introduced in the late 18th century by Edward Jenner when he used cowpox virus as a vaccine to protect humans against smallpox virus infections. This led to the development of vaccines over the next 2 centuries to provide protection against various bacterial and viral pathogens. Undoubtedly, the effective vaccination against infectious diseases is the best method of reducing suffering of human and animals caused by viral, bacterial, and parasitic infections. Over the last 200 years, the technology of vaccine development and production has not changed significantly. This usually involves the use of either a killed pathogen combined with an adjuvant or a live pathogen with reduced virulence. Apart from the tremendous success of killed and attenuated virus vaccines over the years, many of such vaccines do not provide satisfactory protection, and there are a number of other disadvantages associated with these vaccines. Additionally, there are important pathogens against which attempts to develop effective vaccines using traditional approaches were unsuccessful. Various protective viral antigens (envelope and/or capsid proteins or glycoproteins and other viral proteins) and bacterial antigens (surface, internal, or fimbria proteins; bacterial polysaccharides; bacterial toxins; and other proteins involved in bacterial metabolism) have been identified as potential vaccine candidates. These protective antigens are used by various means to develop effective vaccines. The field of vaccine technology is not limited to infectious diseases but 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.

### Conventional Vaccines

#### Inactivated vaccines

Inactivated (killed) pathogenic organisms can be used in vaccines. This is the simplest way to produce vaccines, provided the organisms can be cultured easily. Therefore, this method is often first tested to develop a potential-vaccine. As with any other technique of vaccine production, this procedure is only good for some organisms. There are a number of methods of inactivating pathogenic organisms; the most common are treatment with chemicals (formalin, formaldehyde, or propiolactate), heat, or $\gamma$-irradiation. In some instances, the procedure of inactivation may enhance antigenicity of some antigens important in protection. Inactivated vaccines usually result in good humoral immune response after multiple inoculations. Because inactivated vaccines in general fail to elicit effective mucosal and cell-mediated immune responses, they may provide limited protection against mucosal and intracellular pathogens. Failure to inactivate the pathogenic organisms completely could result in disease instead of protection. During the 1950s, some lots of poliovirus vaccine were not inactivated completely (1, 2). Now, the methods used to detect residual infectivity are more stringent, therefore, inactivated vaccines are considered safe with extremely low or no chance of infection.

There have been instances in which inactivated vaccines led to atypical disease or enhanced disease severity. For example, in the 1960s, formalin-inactivated respiratory syncytial virus (RSV) vaccine actually enhanced the disease symptoms when vaccinated children were naturally exposed to RSV (3, 4). It was later discovered that a change in the antigenicity of RSV F and G glycoproteins (5) resulted not only in alteration in humoral immune response but also in the Th1 and Th2 components of the CD4+ T-cell response to RSV (6).

#### Live attenuated vaccines

Mostly attenuated organisms are being used as live virus vaccines; however, in some instances, even virulent organisms could be used, provided they are not administered via the natural route of infection. For example, human adenovirus types 4 and 7 may cause acute respiratory infections in humans when administered via the oronasal route but provide protection when given orally in enteric-coated capsules (7).
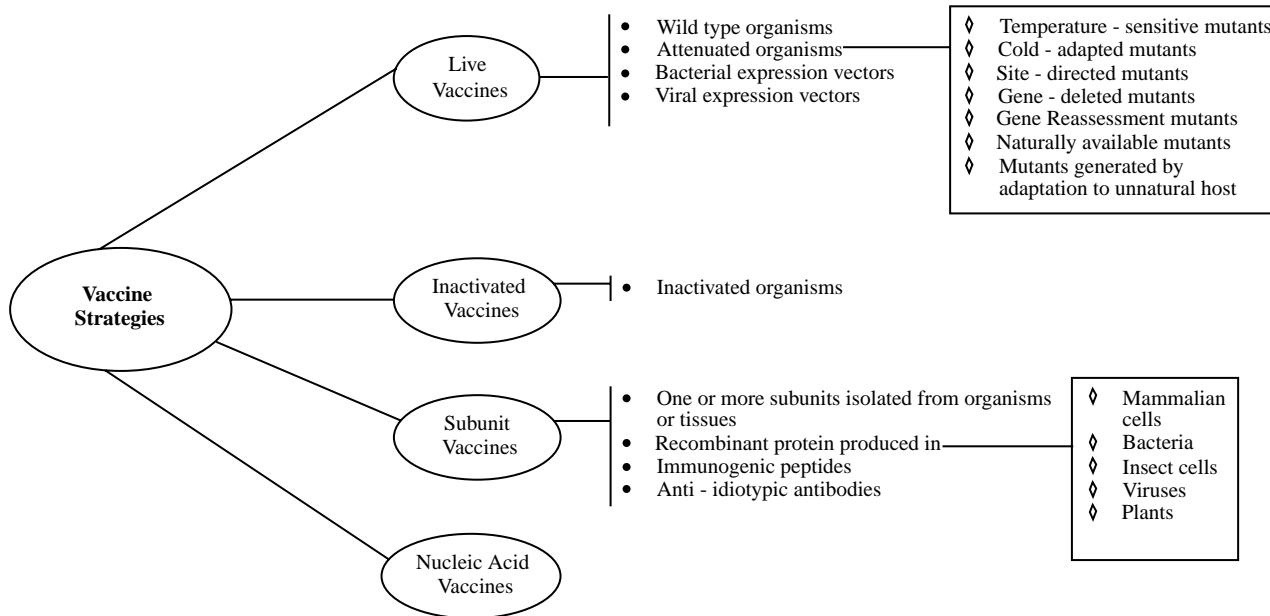
**Fig. 1**   Overview of vaccine strategies.

There are different ways to attenuate pathogens for vaccine production. Attenuation of organisms can be achieved by growing them under abnormal conditions, which include cultivation in unnatural hosts or cell lines. Some organisms are attenuated when they replicate at different pH levels and/or temperatures. In cells infected with multiple viruses with a segmented genome (e.g., influenza virus, reovirus), genome segments are randomly recombined in the progeny. This process of recombination is known as reassortment and is also useful in generating attenuated viruses. A natural pathogen of one host may be attenuated for another host, e.g., vaccinia virus worked as an attenuated vaccine for small poxvirus eradication program during the 1960s and 1970s, and turkey herpesvirus works as an attenuated vaccine for Marek's disease virus (a chicken herpesvirus). In an inoculated host, the attenuated organism replicates without causing disease symptoms, thereby leading to induction of immune response somewhat similar to the natural infection with the disease-causing organism. The Bacille Camet–Guerin (BCG) strain of *Mycobacterium tuberculosis* was attenuated after more than 200 passages on media containing increasing amounts of bile. The Sabin poliovirus vaccine was attenuated by a number of passages in monkeys and in monkey kidney epithelial cells (8). Measles virus was initially adapted to monkey kidney cells and subsequently attenuated in duck embryo and human tissue culture cell lines (9–11).

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 in the nucleotide sequence of a gene so that the resulting protein product of the gene is unable to assume or maintain its functional configuration at the nonpermissive (37–39°C) temperature. The protein, however, is able to assume a functional configuration at the permissive temperature (32–34°C), e.g., herpesviruses, adenoviruses, and influenza viruses. Thus, these mutants can replicate in mucosal sites with a lower temperature, e.g., the nasal cavity, but are unable to cause systemic infections and disease.

A number of advantages associated with live vaccines are that: 1) they are cheap to produce because the inoculum dose is relatively less, 2) they require fewer inoculations, 3) they do not require adjuvants, 4) they elicit both humoral and cell-mediated immune responses, and 5) they can be inoculated by the natural route of infection. Some of the disadvantages associated with live vaccines are that 1) they are usually less stable than inactivated vaccines and may require refrigeration for storage, 2) some of these vaccines under certain situations may revert to virulent form in the host and thereby lead to clinical disease, 3) they may not be recommended for immunosuppressed, immature, older, or pregnant hosts, 4) they may have a low level of residual virulence, and 5) they may be contaminated with other adventitious organisms.

## Recombinant Vaccines

### Recombinant vector vaccines

**Viral vectors:** 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. The route of vaccine delivery also plays an important role in determining the type of resultant immunity induced. A number of viruses, such as adenoviruses, poxviruses, herpesviruses, picornaviruses, togaviruses, orthomyxoviruses, paramyxoviruses, and others, have demonstrated considerable potential as vectors for antigen delivery at mucosal surfaces (12). Immunogenic foreign epitopes can be expressed on the virus surface by modifying the viral capsid or envelope protein (13). A wide variety of foreign viral antigens has been expressed in viral vectors, and vaccination-challenge studies in experimental animals have demonstrated moderate to complete protection. Immunization with such vectors leads to the foreign viral antigen expression similar to that of natural infection without causing disease. Antigenic peptides are expressed along with major histocompatibility (MHC) class I and class II antigens and, thus, result in both humoral and cytotoxic T-cell responses.
Both adenovirus-and poxvirus-based vectors have a number of common advantages including that 1) vector construction is easy, ii) relatively high levels of foreign protein expression are easily attained, 3) relative thermostability, 4) they have a large capacity for foreign DNA insertion, 5) vector derivatives are nonpathogenic, and 6) they have a wide host range. More than one foreign antigen can be expressed in the same vector to provide protection against a number of diseases by inoculation with a single vector.

Vaccinia virus expressing rabies glycoprotein has been licensed for use to control rabies in the wildlife population, especially raccoons, foxes, skunks, and coyotes (14). Baits containing a live vaccinia-rabies glycoprotein recombinant virus vaccine are distributed in the rabies endemic area with the intention that rabies-susceptible wild animals that eat these baits will become immunized against rabies virus (15), and this approach has demonstrated satisfactory results. Vaccinia virus expressing the F and H gene of rinderpest virus has shown potential for its use to control rinderpest in developing countries (16, 17).

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 that leads to foreign antigen expression similar to replication-competent vectors. Replication-defective adenovirus vectors are generated by deleting the early region 1 (E1) genes (18,19). E1-deleted vectors can be grown in an E1-complementing cell line, and animals immunized with such vectors elicit a protective immune response (20). Avian poxviruses grow normally in avian cells but would result in an abortive infection in mammalian hosts. Dogs and cats immunized with an avipox-rabies glycoprotein recombinant are protected against rabies virus infection (21).

**Bacterial vectors:** Similar to viral expression vectors, attenuated bacteria can be developed as vectors for foreign gene expression and delivery for the purpose of multivalent vaccines. Immungenic foreign epitopes can be expressed on bacteria surfaces by modifying cell surface proteins, fimbria, or flagella. It has been demonstrated that *M. bovis* BCG strain induces both strong humoral and cell-mediated immunity, therefore, it has been developed as a delivery vector with the assumption that foreign proteins expressed by *M. bovis* in inoculated individuals will also raise a strong protective immune response (22). Because *Salmonella* and *Vibrio* colonize in the intestinal tract, attenuated strains of these bacteria were developed as vectors for mucosal delivery (23–26).

Various bacterial vectors have been used to express a number of bacterial *(B. pertussis, S. pneumoniae, Y. pestis, and L. monocytogenes)*, viral (herpesvirus, influenza virus, human immunodeficiency virus, simian immunodeficiency virus, and hepatitis B virus), and parasitic (*S. mansoni*, and *L. major*) antigens (26). Significant improvements in attenuation of bacteria, and the stability, localization, and expression levels of heterologous antigens are required to market the bacterial vector-based vaccines for use in humans or animals.

To enhance foreign gene expression, "balanced lethal," plasmid-based expression vehicles have been developed (27). A foreign antigen may form inclusion bodies or localize in intracellular compartment of the vector thereby affecting the type, levels, and duration of immune response elicited against the antigen. The *Escherichia coli* α-hemolysin secretion system (HSS) that includes HlyB, HlyD, and TolC is involved in exporting the HlyA-fused foreign antigens to extracellular compartment (28). Using the HSS system for attenuated *Shigella dysenteriae*, the expression and secretion of Shiga toxin-B subunit were obtained (29).

### Gene-deleted vaccines

Many attenuated vaccines are derived after introduction of random mutations in the genomes of various pathogens. In

situations in which these random mutations may be point mutations, attenuated organisms may regain virulence owing to back mutations. Because of our increased understanding of virulence of various pathogens at the molecular level, one or more genes responsible for virulence has been identified in many pathogens. The genes associated with virulence may be genes involved with nucleic acid replication and other nonstructural and structural components of the organism. This has made it possible to delete one or more of these genes involved in virulence—another strategy to produce safer attenuated vaccines.

Pseudorabies virus has been attenuated by deleting genes associated with viral virulence. These genes include the thymidine kinase gene (nonstructural protein) involved in viral DNA replication and the gC, gG, and gE genes (nonessential glycoproteins) involved in virus assembly (30, 31). A gene-deleted vaccine of pseudorabies virus has proved highly effective in controlling this viral infection under field conditions. It has been demonstrated that *Salmonella typhimurium* aroA, aroB, and aroC deletion mutants fail to grow in its 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 at least for a short period in its host, thus raising a protective immune response (32). 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 (33, 34). 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.

## Subunit vaccines

A subunit vaccine consists of one or more immunogenic epitopes, proteins, 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 (35, 36). The pathogen could be disrupted, and one or more immunogenic proteins such as bacterial cell wall proteins; flagella or pili; and viral envelope, capsid, or nucleoproteins can be purified. 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.

A number of expression systems including bacteria, yeasts, mammalian cells, insect cells, and plants are now available for foreign protein expression. High amounts of a foreign protein can be produced in a bacterial-expression system at a low cost. Because scale-up and downstream processing have been well worked out for bacterial-expression systems, they are usually first tested for subunit vaccine production. Many of the immunogenic proteins, especially of viral origin, require secondary modifications that are important for their antigenicity. A bacterial-expression system may produce proteins of altered immunogenicity because the bacterial system lacks many posttranslational processes. However, some viral glycoproteins expressed in bacteria induce protective immunity, e.g., the gp 70 gene of feline leukemia virus (37). A yeast-expressed hepatitis B virus surface antigen (HbsAg)-based subunit vaccine is currently in use for humans and has demonstrated excellent protection against hepatitis B virus infection (38). This vaccine is an excellent example of the potential of recombinant subunit vaccines for providing protection against many viral and bacterial infections.

Because mammalian cells are known to process viral glycoproteins to their functional form by secondary modifications, they are considered one of the means to produce viral antigens for subunit vaccine production. However, the expression of such proteins in mammalian cells is usually too low. It was demonstrated that the 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 could be used as a subunit vaccine without further purification. However, the removal of transmembrane anchor may potentially alter antigenicity of the secreted protein. A number of viral glycoproteins that were expressed either in mammalian or in insect cells and secreted in form of proteins were suitable for providing protective immune response include F and G genes of respiratory syncytial virus (39), the HN and F genes of parainfluenza virus (40), and the gD gene of bovine herpesvirus type 1 (41).

**Immunogenic antigen production in plants:**  In the past decade, 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 (over 90%) gain of plant biotechnology will result from the use of plants as bioreactors to produce high-valued products such as vaccines, industrial enzymes, and other pharmaceuticals.

Production of subunit vaccines in mammalian cells is usually expensive because of the low level of foreign gene expression and high processing cost. High levels of foreign gene expression can be obtained in bacteria and yeast, but many animal viral or mammalian 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. Because most mechanisms regulating secondary modifications of proteins are present in plants, transgenic plants offer an attractive alternative to produce functional viral, bacterial, or parasitic proteins in large quantities at a very low cost for subunit vaccine production (42). Similarly, the production of functional multimeric antibody molecules in plants has made it possible to manufacture antibodies in bulk amounts for passive immunization (43).

Two major strategies have been devised to produce foreign proteins in plants. These are: 1) the stable integration of chimeric gene into the plant genome under a suitable constitutive or inducible plant promoters (44, 45), and 2) manipulation of plant pathogenic viruses (46). Foreign protein expression in plants usually range from 0.01 to 1% of the total plant protein.

The hepatitis B virus (HBV) surface antigen HBsAg produced in transgenic tobacco elicits an immune response when injected in mice (47). Mice fed transgenic potato tuber expressing B subunit of heat-labile enterotoxin (LT-B) of enterotoxigenic *E. coli* developed antibodies to LT-B, particularly IgA antibodies (44). Dalsgaard et al. (46) 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 against challenge with virulent foot-and-mouth disease virus (FMDV) in mice inoculated with the structural protein VP1 of FMDV produced in transgenic *Arabidopsis* has been shown (45). It has been hypothesized that transgenic plants could serve as "edible vaccine," thereby providing a very inexpensive mean of oral immunization (48).

### 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 in 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 mimic the epitope of immunizing antigen and 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 antigen. Anti-idiotype vaccines are useful in cases in which 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 (49, 50). This type of vaccine is still in the developmental stage.

### DNA Vaccines

Immunization of mammalian hosts with a plasmid DNA containing a gene under control of a heterologous promoter has introduced a new approach in the area of recombinant vaccine design. The introduced DNA is taken up by 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. DNA vaccines can also be called polynucleotide vaccines or nucleic acid (NA) vaccines. Such vaccines appear to have the primary 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 NA vaccine evolved from initial studies in experimental animals in which the inoculation with naked plasmid DNA resulted in a protective immune response (51). 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,
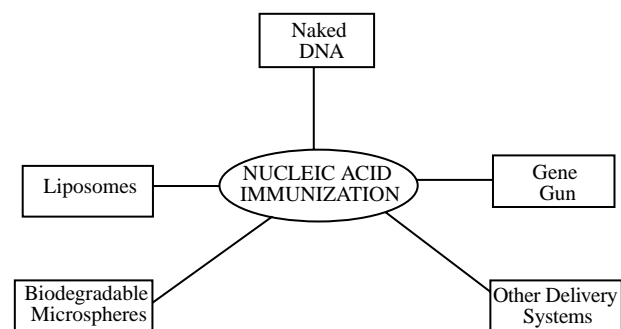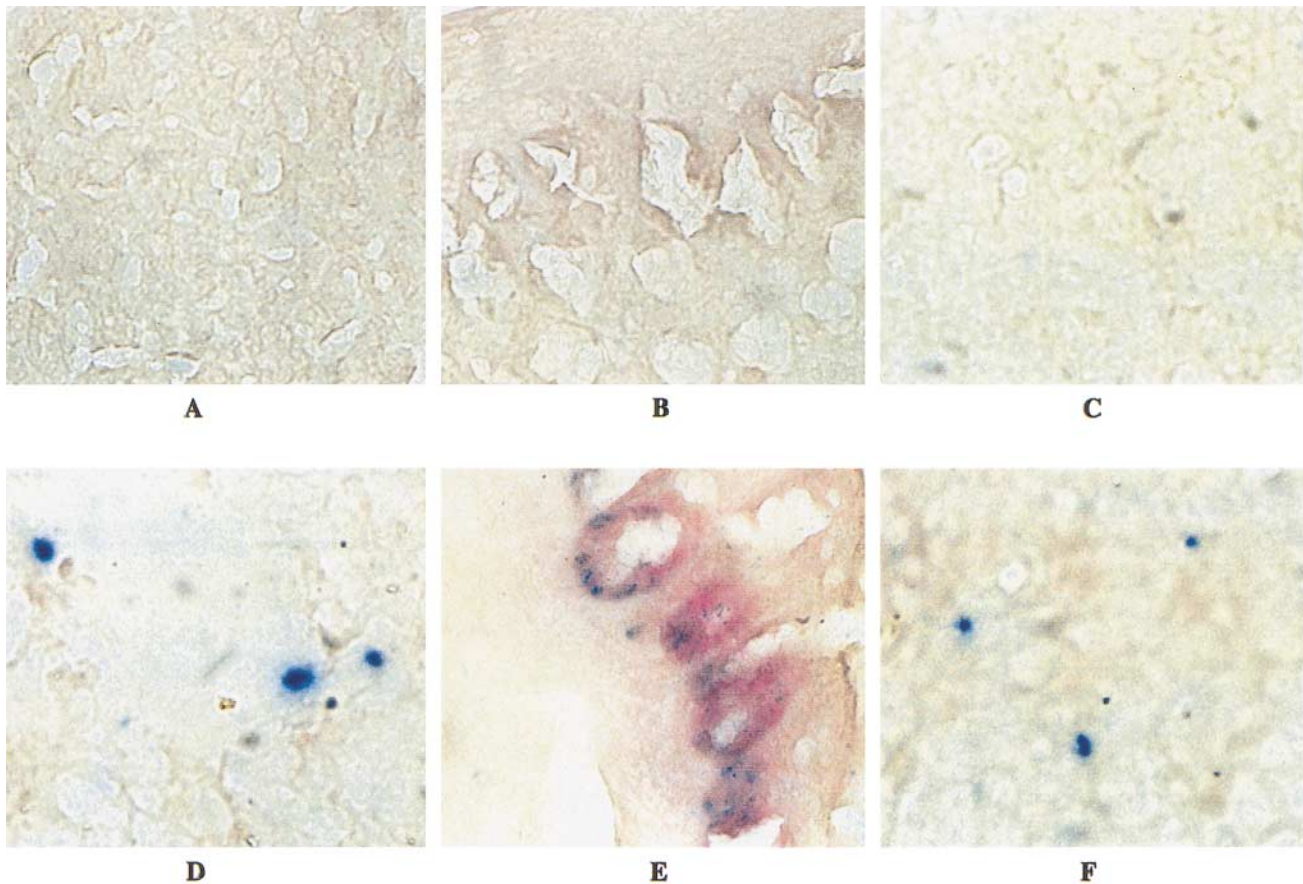


**Fig. 2** Methods of nucleic acid delivery.

**Fig. 3** β-Galactosidase expression in tissues of mice inoculated orally with alginate microspheres containing plasmid DNA: (A) liver, (B) intestine, and (C) spleen sections from the animal inoculated with microspheres containing bovine adenovirus type 3 (BAd3). (D) Liver, (E) intestine, and (F) spleen sections from the animal inoculated with microspheres containing LacZ plasmid +, BAd3. (From Ref. 57.)

such as bombardment with gold microparticles coated with NA (52, 53), incorporation of NA into liposomes and other polycationic lipids (54, 55), biological erodable polymers (56), and others, have been developed (Fig. 2). Recently, it has been demonstrated that alginate microspheres can be used for the encapsulation, delivery, and expression of plasmid DNA (57) (Fig. 3). 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 (58). 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, SIV, HTLV-1, influenza virus, hepatitis B virus, hepatitis C virus, herpesvirus, M. tuberculosis, *Leishmania,* malaria, and many more have been expressed by NA vaccines and have demonstrated encouraging results (59–63).

## Adjuvants

Adjuvants are compounds that, when administered in combination with antigens, enhance the immune response to those antigens. This enhanced immunogenicity can be measured as an increase of antigen-specific antibody levels in serum and/or mucosal secretions, a response against an increased number of epitopes, an increase of cell-mediated immune responses, or a combination thereof. Adjuvants

are particularly important for the induction of protective immune responses against weak immunogens such as subunit vaccines. The mechanisms by which adjuvants enhance the immunogenicity of antigens are not completely understood, but they include immunostimulation, altered processing of antigens, and sustained release of antigens (depot effect). A different type of immune response is obtained by administration of antigens via the oral route, and this has different delivery requirements.

Many compounds can act as adjuvants. Their classification is made difficult by the variety in chemical composition and the overlapping, often poorly understood, mechanisms of action. Only aluminum adjuvants are approved by the FDA for use in human vaccines. Quil A is a saponin that is commonly used as an adjuvant in veterinary vaccines and is also a component of immune-stimulating complexes (ISCOM). These adjuvants are addressed in some detail below. A detailed discussion of other types of adjuvants can be found in recent books (64–66) and reviews (67) on this subject.

## Immunostimulation

The immune system can be divided into the adaptive immune system, comprising of B and T lymphocytes, and the innate immune system, which includes neutrophils, macrophages, dendritic cells, and soluble factors such as the complement system. The innate immune system plays a critical role in the activation of the adaptive immune system. Dendritic cells are antigen-presenting cells that integrate the signals from the innate immune system and activate T-cells and possibly B-cells. T-cells have antigen-specific receptors that recognize peptides displayed by MHC I molecules (CD8$^+$ cytotoxic T-cells) and MHC II molecules (CD4$^+$ T helper cells). Engagement of the antigen-specific T-cell receptor is not sufficient, and T-cells also need to receive costimulatory signals delivered via CD28 and CD40-ligand. Dendritic cells express both MHC I and MHC II and, on activation, increase the expression of the costimulatory molecules CD80 and CD86 (ligands for CD28) and CD40. The signals that activate dendritic cells include microbial molecules. The innate immune system is equipped with receptors (called pattern-recognition receptors) that can recognize molecules that are expressed by pathogens, but not by mammalian cells, and alert the innate immune system on infection. These molecules, pathogen-associated molecular patterns, include lipopolysaccharides (LPS), mannose, and bacterial DNA with unmethylated CpG motifs. In addition, dendritic cells are stimulated by host cell components that are expressed and/or released by cells when they undergo stress and pathologic cell death (necrosis). The identity of these components, called danger signals, is uncertain but

may include heat shock proteins. The microbial molecules and danger signals can directly activate dendritic cells, or they can activate other components of the innate immune system resulting in the secretion of cytokines and other mediators that activate dendritic cells. The activated dendritic cells, in turn, activate T- and B-cells.

Immune responses can be divided into type 1 and type 2, based on the pattern of cytokine secretion and functional outcome of the immune response. Type 1 immune responses are characterized by secretion of IFN-gamma, production of IgG2a in mice, and activation of macrophages, NK cells, and cytotoxic T-cells. Type 2 responses are characterized by secretion of IL-4, IL-5, and IL-13 and by IgG1 and IgE production. The responses are reciprocally regulated. How the polarization of the immune response toward type 1 or type 2 is determined is not exactly understood. IL-12 is an important factor that drives the type 1 response, and IL-4 is implicated in the type 2 response. Microbial products such as LPS and bacterial DNA stimulate the secretion of IL-12 by dendritic cells and preferentially induce type 1 immune responses.

It is likely that the primary mechanism by which adjuvants stimulate the immune response is by direct or indirect signaling through pattern-recognition and danger signal receptors. Very strong adjuvants are often composed of or include microbial components such as LPS and mycobacteria or derivatives thereof. These type of adjuvants bind to pattern-recognition receptors to stimulate IL-12 production and a type 1 immune response. Coadministration of cytokines can directly activate and influence dendritic cells and the outcome of the immune response. This was clearly demonstrated with an experimental Leishmania vaccine using IL-12 as an adjuvant. Immunization of genetically susceptible BALB/c mice with a Leishmania antigen did not result in protection, but when IL-12 was injected with the antigen, the mice became markedly resistant to infection. The effect of IL-12 correlated with increased IFN-$\gamma$ and decreased IL-4 secretion by antigen-specific T-cells in vitro.

## Altered processing of antigens

Most T-cells that carry the $\alpha-\beta$-T-cell receptor do not recognize and react with intact proteins. Instead, the T-cells recognize small peptides that are derived from proteins and that are linked to MHC I and MHC II molecules. The MHC I-linked peptides are generated in the cytoplasm (endogenous pathway) and recognized by CD8$^+$ T-cells. Proteins in the cytoplasm are degraded by a complex of proteolytic enzymes, the proteasome, and the peptides are transported into the rough endoplasmic reticulum where they associate with MHC I molecules. Peptide binding

stabilizes the MHC I molecules, and the complexes are transported to the cell surface. In contrast, proteins that enter cells by endocytosis are partially degraded into peptides in endosomal vesicles. The peptides bind MHC II molecules that have been transported from the endoplasmic reticulum to the endosomes. The MHC II–peptide complexes are then displayed on the cell surface and are available for recognition by CD4$^+$ T-cells.

Vaccines that contain single proteins or inactivated pathogens can readily activate CD4$^+$ T-cells because the antigens are endocytosed and processed by MHC II–positive antigen-presenting cells. Activation of the CD4$^+$ T-cells can result in a type 1 or a type 2 immune response, depending on the type of adjuvant included. However, such vaccines usually do not activate CD8$^+$ cytotoxic T-cells because activation of CD8$^+$ T-cells requires processing of antigen via the endogenous pathway. Certain adjuvant formulations such as liposomes, the saponin QS-21, and poly-(lactic-*co*-glycolic acid) (PLGA) are able to induce cytotoxic T-cell responses to protein antigens (68). These adjuvants appear to target some of the injected antigens into the cytosol of antigen-presenting cells for processing via the endogenous pathway. The mechanism by which this occurs is not known.

## Sustained release of antigens

The slow and continued release of antigens has been postulated to induce a strong immune response through continued activation of the immune system. This may contribute to the adjuvant effect of aluminum-based adjuvants and mineral oils. Newer technologies may allow for the design of vaccines that release antigens from a depot at certain time intervals after a single injection. One example is the use of poly PLGA microspheres for encapsulation of antigens. By varying the polymer composition and size of the microspheres, the release of antigen can be varied. Pulsatile release of antigen can be attained by combining multiple variations of PLGA microspheres in a single dose of the vaccine (69). Relatively little is known about the desired pattern of antigen release to obtain a maximal response. It was recently suggested that continued release of antigen is not desirable for the induction of strong memory cell responses. Mathematical models may help design appropriate strategies for the release of antigens from depots after a single injection (70).

## Aluminum

Aluminum adjuvants in human vaccines are either aluminum hydroxyphosphate (commonly referred to as aluminum phosphate) or aluminum oxyhydroxide (aluminum hydroxide) (71). Aluminum-based vaccines are prepared by adsorption of antigen to commercial aluminum hydroxide or aluminum phosphate gels or by mixing antigen with alum (potassium aluminum sulfate), resulting in precipitation. The alum-precipitated adjuvants resemble aluminum phosphate in their chemical and physical properties (71). The surface charge and morphology of the aluminum adjuvants affect their adsorptive capacity. The rate and degree of adsorption are further dependent on the pH, ionic strength of the antigen solution, and isoelectric point of the antigen.

Aluminum adjuvants are universally used in diptheria–tetanus–pertussis (DTP) vaccines and in most hepatitis B vaccines and have an excellent safety record. They are not ideal adjuvants, however, because the enhancement of the immune response is relatively weak, they are not effective with all antigens, and, most important, they only enhance the humoral (type 2) immune response and have little effect on the cell-mediated (type 1) immune response.

The mechanism by which aluminum enhances the immune response is not clear. Early studies suggested that aluminum adjuvants slowly release the adsorbed antigen over time (depot effect). However, recent experiments demonstrated that antigens are rapidly desorbed after injection in animals. Moreover, aluminum phosphate enhanced the immune response to DNA-encoded antigen after DNA immunization, clearly indicating that adsorption may not be critical to the adjuvant effect of aluminum compounds. These data indicate that aluminum enhances the immune response via other mechanisms. A satisfactory explanation of the adjuvant effect of aluminum also needs to take into account its selective mode of enhancing the immune response, i.e., a predominant type 2 immune response. Aluminum adjuvants induced differentiation toward type 2 immune responses, even in the absence of IL-4 or IL-13. Aluminum stimulated a type 1 and type 2 immune response in genetically engineered mice with a defective IL-4 and IL-13 response, suggesting that aluminum-induced IL-4 and/or IL-13 secretion suppresse the type 1 response but are dispensable for a type 2 response in intact animals (72). The lack of a type 1 immune response is a drawback for the use of aluminum in vaccines for intracellular pathogens and tumors. A recent study demonstrated that aluminum adjuvant with adsorbed IL-12 induces a strong type 1 response, indicating that it is possible to overcome the aluminum-induced suppression of type 1 responses (73).

## Saponins

The saponins of the bark of the *Quillaja saponaria* Molina tree have long been known to have immunostimulatory activity. A partially purified fraction, Quil A, has reduced

toxicity and more potent adjuvant activity and is used in veterinary vaccines. Quil A can be further fractionated into fractions that have different degrees of toxicity. QS-21 is a less toxic component with strong adjuvant activity. Saponins probably act by direct stimulation of the immune system (74). They 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. Cytokine analysis indicates that QS-21 stimulates type 1 cytokine production.

Immune-stimulating complexes (ISCOMs) are 30–40 nm particles consisting of Quil A, cholesterol, antigen, and phospolipids (74). They are used in a commercial vaccine for equine influenza. ISCOM-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. As with Quil A, ISCOMs target antigens for processing via the MHC I pathway, resulting in induction of cytotoxic T-cell responses.

## Delivery of Vaccines

### Parenteral versus mucosal route

The success of vaccination depends primarily on the method of presenting the antigen to the host immune system. Antigens have usually been delivered by parenteral (such as intravenous, intramuscular, intraperitoneal, intradermal, and subcutaneous) administration, but recent studies have shown that other routes of delivery such as intranasal, oral, and transdermal delivery have also been effective. In some cases, vaccination through mucosal routes resulted in better responses in IgA production. Because nonparenteral vaccine delivery presents many obvious advantages, numerous attempts have been made on the development of nonparenteral delivery of vaccines.

**Parenteral route:** Parenteral vaccination remains the immunization method of choice for most antigens because it provides more effective immune response than do any other routes of vaccination in most cases. Every years millions of people receive inactivated influenza vaccine by parenteral administration. Subcutaneous vaccination with inactivated influenza vaccine is known to induce simultaneous immune responses in the blood and upper respiratory tract of subjects. The immune response, i.e., the increase in the number of influenza virus-specific antibody-secreting cells in peripheral blood and tonsils, increased rapidly to reach a peak within 1 week after vaccination (75). Parenteral vaccination of a DNA vaccine encoding glycoprotein D of herpes simplex virus type 2

resulted in systemic cellular and humoral responses. The mucosal humoral responses generated by intramuscular and intradermal vaccination were comparable with those 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 (76). For many other antigens, however, the usefulness of parenteral vaccination is limited by the insufficient induction of mucosal immune responses.

Parenteral vaccination is difficult for those living in the developing countries where medical care is not well-established. Vaccination of a large number of subjects using hypodermic needles, which is a highly labor-intensive procedure requiring healthcare personnel, is not practical. The problem becomes even more significant for vaccination of millions of animals. For example, vaccination for routine control of Newcastle disease in chickens by intramuscular injection requiring individual handling of the birds is not practical (77). Recent advances in needleless injectable systems have made the parenteral vaccination easier, but it still requires individual handling. Examples of needleless injection systems are Powder-Ject®, 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 (78). 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. Delivery to the skin by the gene gun induced exclusively IgG1 antibodies (Th2-like) at 4 weeks and only very low IgG2a levels at later times. Other routes, such as intraperitoneal, intraperineal, subcutaneous, intranasal inhalation, intranasal instillation, intrarectal, intravaginal, ocular, and oral, did not result in significant immune responses (79).

*Dual-chamber syringe.* For delivery of two established vaccines (e.g., polyribosyl ribitol phosphate conjugated to tetanus toxoid and diphtheria–tetanus–whole cell pertussis and inactivated poliovirus vaccine) at the same time, a dual-chamber syringe delivery system can be used. The proximal chamber may contain 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 (80). The primary advantage of the dual-chamber syringe is that it reduces the cost of vaccine delivery and, at the same time, increases the vaccine acceptability and coverage rate of vaccines (81).

**Mucosal route:** Vaccination through mucosal routes provides new avenues of vaccination with a unique advantage of mucosal immunity, that may not be obtained, through parenteral vaccination. Mucosal immunization presents a realistic alternative to parenteral administration for inducing protective immune responses. Vaccination by mucosal route provides a number of advantages over parenteral vaccination. First, mucosal vaccination does not involve hypodermic needles, which are not user-friendly. Second, the total surface area of the mucosal surfaces in the gastrointestinal, respiratory, and urogenital tracts where many infectious pathogens come into contact with the host is huge. Thus, preventing infections at the mucosal surface

provides an immunological first line of defense against diseases (82). This makes priming of the mucosal-associated lymphoid tissue (MALT) by vaccination most desirable. Parenteral vaccination alone 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 (83). The mucosal tissues are protected by interconnected local immune system, which is essentially separated from systemic immunity (84). 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 reaction in other mucosal tissues. It is known that the mucosal immune system produces 70% of the body's antibodies (85). Fig. 4 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
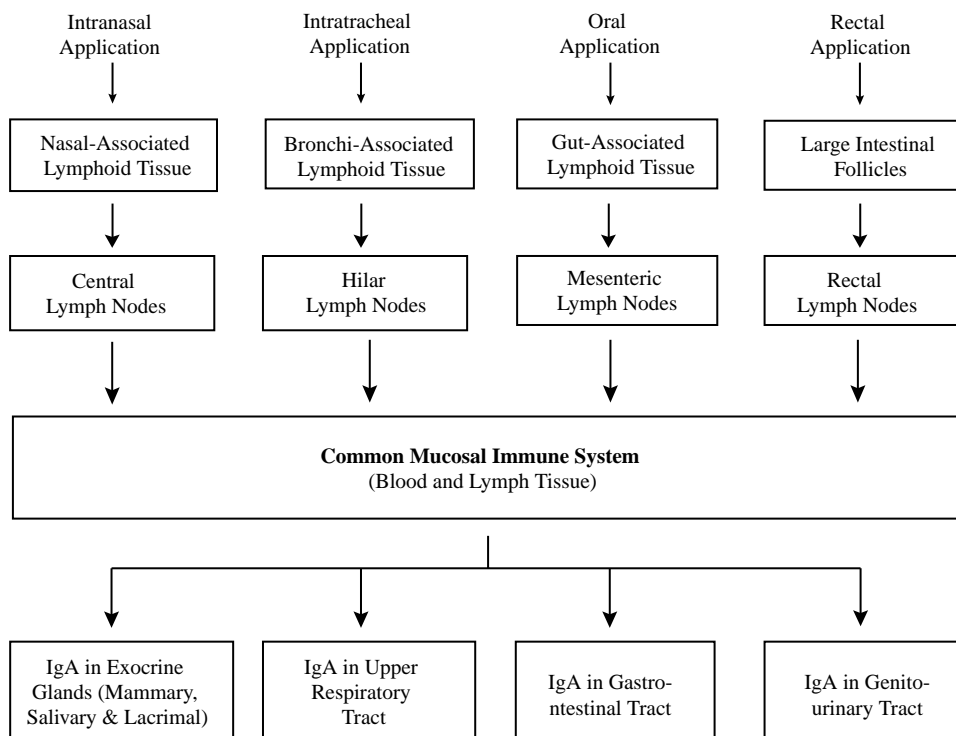


**Fig. 4** Mucosal immunization and production of IgA antibodies in various mucosal surfaces via the common mucosal-simmunization system. Nasal and rectal vaccinations usually result in IgA production in upper respiratory tract and genitourinary tract, respectively, whereas effector sites by oral vaccination are expected to include many mucosal surfaces.

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 (86).

*Oral vaccination* of the various mucosal routes, oral vaccination is the most preferable mode of vaccination because of its ease of use and low cost of manufacturing (87). Furthermore, the gastrointestinal (GI) tract provides the largest component of the mucosal immune system that has been well-characterized. Oral administration of vaccines has high acceptability, by avoidance of injection, to individuals of all ages. Fig. 5 shows the current understanding of oral vaccination. 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 the macrophages and B-cells (B). These cells in turn present the antigen to T helper lymphocytes. These cells migrate into the blood via the mesenteric lymph nodes (MLN) and the thoracic duct (TD). 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.

The maximal intestinal immunization can be achieved by intra-Peyer's patch immunization, and thus this method can be used to screen oral vaccine candidate antigens without the added complication of simultaneously testing
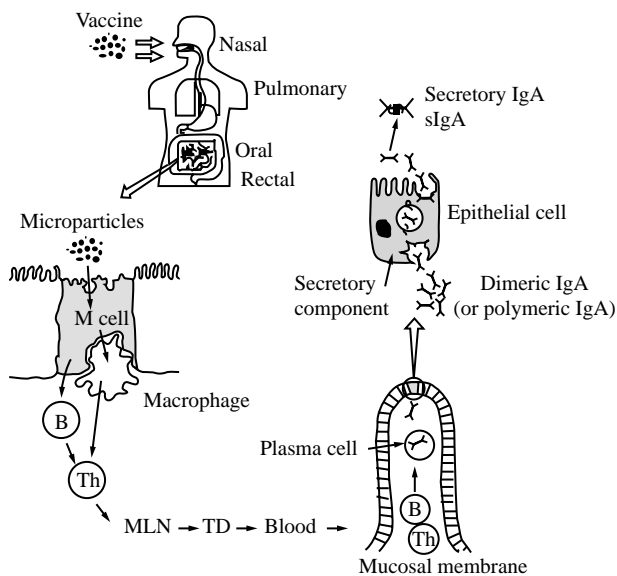


**Fig. 5** Mucosal immunization by oral vaccination.

oral-delivery systems (88). Immunization of subjects against *Helicobacter pylori* by intra-Peyer's patch resulted in an 84–91% reduction in *H. pylori* infection compared with unimmunized controls. The therapeutic efficacy of the recombinant *H. pylori* urease vaccine in mice was shown to be comparable with that achieved with the combined antibiotic/antacid treatment in humans. The oral vaccination is preferred to conventional treatment of ulcers because it is a very simple and quick procedure compared with long-term conventional treatment. In addition, vaccines use the defense mechanisms of the body to establish long-lasting immunity (89).

One of the limitations of oral vaccination is that it does not always induce sufficient immunity. There are a few good reasons for this. First, the GI tract is designed to digest proteins by acidic and enzymatic degradation for absorption. 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 important 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 very poor. Even after oral intake of gram quantities of antigen, only a nanogram range of antigenic material was found to pass the intestinal barrier (90). It is also possible that for certain antigens, oral vaccination may simply be less effective than parenteral vaccination in induction of systemic immunity (91). The protection resulting from oral vaccination is known to last for a relatively short period, ranging from a few months to 1 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 (also called microcapsules or microspheres). Antigens that are encapsulated in microparticles are protected from degradation, and the microparticulate nature allows better 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. It is noted here that virus itself 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 (92). Influenza virus can also elicit immune response after oral administration. 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 (93). The exact relationships between the surface chemistry and the uptake by Peyer's patches, however, have not been fully understood. Development of better oral vaccines requires understanding of such relationships.

*Intranasal vaccination* route has received growing interest for noninvasive immunization. Intranasal immunization has been quite effective for various vaccine-delivery systems. Both solution and microsphere formulations tend to show good immune responses after intranasal administration. Immunization of mice with tetanus toxoid, in solution and microsphere-encapsulated formulations, resulted in high levels of specific IgG and IgA antibodies (94). Nasal vaccine delivery is known to be superior to oral delivery in inducing specific IgA and IgG antibody responses in the upper respiratory tract (95). 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 (95). It is interesting to note that the volume of the nasally instilled vaccine is important (94). The larger-volume (e.g., 50 $\mu$L) of microsphere suspension resulted in the higher percentage of particles entering the lungs than did the lower, volume (e.g., 10 $\mu$L) instillation.

It is generally believed that microspheres that adhere to the nasal mucus elicit better immune response, and for this reason, many microspheres made of mucoadhesive polymers, such as chitosan, have been used extensively in the preparation of nasal vaccine formulations.

*Transdermal vaccination* or transcutaneous immunization, is attractive, because it does not require 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 was able to achieve localized transgene expression within a restricted subset of skin in mice. It also elicited an immune response against the protein encoded by the DNA (96).

For improved results, transdermal electroporation was also tried to explore the feasibility of nonadjuvant, needle-free skin immunization (97). The transdermal electroporation route elicited higher responses to a myristylated peptide than did intradermal immunization. For diphtheria toxoid, however, the result was the opposite. It appears that transdermal electroporation is a promising technique for nonadjuvant skin immunization, especially with 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 (98, 99). It appears that transcutaneous immunization can induce potent, protective immune responses to both systemic and mucosal challenge (100).

*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 (101). Recent advances in powder inhaler devices have made it possible to deliver vaccines via the pulmonary route using dry powder inhalation technologies (102). Dry powder vaccine in the size range from 1 to 5 $\mu$m in diameter is used for the maximum alveolar (deep lung) deposition (101).

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 (103). Such a complex formation can be considered a formation of microparticles that can enhance cellular uptake and subsequent immune responses.

**Parenteral and mucosal combination vaccination:** The combination of mucosal and systemic immunization routes (e.g., parenteral immunization followed by oral immunization or vice versa) generally induces mucosal immune responses that are superior to immunization by either route alone (91). Pigs showed some protection after intramuscular inoculation with formalin-inactivated *M. hyopneumoniae* vaccine in incomplete Freund's adjuvant and a booster inoculation with the same vaccine in microspheres onto the mucosal surface of Peyer's patches by a surgical operation (104).

## Antigen delivery systems

The primary goal of antigen-delivery systems is to maintain a stable dosage form during storage and, when administered to present antigens to elicit a vigorous immune response in vivo. 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 release or multiple pulsatile release during the desired period would eliminate the inconvenience of multiple vaccine administration for obtaining satisfactory immune responses. The antigen-delivery system plays one of the most crucial roles in the outcome of the immunization. The way that antigens are delivered affects the immune response significantly. Currently, antigen-delivery systems are classified into two systems: live attenuated microorganisms and nonliving microparticulate systems.

**Live attenuated organisms:** Live attenuated bacteria and viruses have been used not only as vaccines but also as a delivery system that elicits humoral, mucosal, and cellular immune responses against exogenous antigens. Since the success with live attenuated oral vaccines against tuberculosis and polio more than 3 decades ago, a number of live attenuated microorganisms have been used as antigen-delivery systems. Live vaccines are relatively easy and cheap to manufacture, because they do not require purification of antigens or formulation with adjuvants (82). Attenuated strains of microorganisms can be formed spontaneously or induced by heat, chemical, or UV mutagenesis. Another advantage of the attenuated live vaccines is that they can be administered by the natural route of infection. Recently, pathogenic microorganisms have been attenuated by genetic engineering, i.e., mutating specific genes or removing some toxic genes. Because much of the infection occurs through the mucosal surfaces, live attenuated vaccines are best suited for protection against pathogens that access the body through the mucosal surfaces. Live attenuated oral vaccines are expected to provide the most convenient and effective means of vaccinating against enteric disease (105). Orally administered attenuated *Salmonella* are known to interact with the MALT (82). Other examples of live attenuated microorganism vaccines are BCG (bacilli Calmette–Guérin), adenovirus, and poliovirus.

Some viruses and bacteria are inherently quite stable. For example, polio virus can be formulated as a frozen liquid. A live poliovirus vector expressing a foreign antigen generates both antibody and cytotoxic T-lymphocyte responses in mice (106). Most live bacteria and viruses, however, are usually stored as powders after freeze-drying or lyophilization. Preserving the live state through freeze-drying often requires the presence of a stabilizer, which is selected primarily through trial and error. The most widely used nonspecific stabilizers are sugars, amino acids, polyols, and neutral salts which are known to act as bound water substitutes for maintaining the conformational integrity of proteins. An example of lyophilized vaccine products is *S. typhi* bacteria lyophilized to a powder that is encapsulated into gelatin for oral administration (107).

One of the drawbacks of using live microorganisms is that attenuated pathogens may invoke the very disease they are designed to prevent if they are insufficiently attenuated. Even if they are sufficiently attenuated, they still may cause severe infections in immunocompromised individuals. In addition, they always have a potential to revert to full virulence if lesions causing attenuation are not fully characterized (82). If pathogens are over-attenuated, they fail to trigger an appropriate immune response. Thus, it is highly important to attain the right balance between minimal virulence and maximal immunogenicity. This balance can be achieved in a normal population but may not be the same in a population with even minor defects in immune competence (108) Another aspect to notice in using live vaccines is that the distribution of live vaccines requires a cold chain that may not be readily accessible in many developing countries, and this may offset advantages of using live-vectored vaccines (109).

**Nonliving microparticulate delivery systems:** Nonliving immunogens generally result in immune responses of lesser magnitude and of shorter duration than do those by living immunogens (82). Nonliving immunogens are usually made of microparticulate forms to protect antigens and to improve cellular uptake. Nonliving microparticulates that can be used as antigen-delivery systems include polymeric microparticles, liposomes, virus-like particles, neosomes, and cross-linked protein crystals. 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 area is usually less than 50 μm (110). 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 the size of microparticles often affect the outcome.

Polymeric microparticles and liposomes have been used extensively as controlled-release dosage forms for many drugs including antigens. They have been quite useful in oral delivery of antigens because encapsulation in microparticles can protect antigens from acidic and enzymatic degradation in the GI tract, and thus serve as a stable vaccine vehicle with extended shelf life. Delivery of antigens by microparticulate-delivery systems has the potential benefits of reducing the number of inoculations, enhancing the immune response via both parenteral and oral vaccination routes, and reducing the total antigen dose required to achieve immune protection (111). Microparticulate vaccine-delivery systems show improved immune responses because of the protection of the loaded antigens from degradation and the slow release of the

antigens. For this reason, microparticulate-delivery systems are often considered adjuvants (66).

*Polymer microparticles,* a large number of polymers, such as poly(methyl methacrylate), poly(butyl cyanoacrylate), poly(lactide-*co*-glycolide), polyarcylstarch, dextran, albumin, and alginic acid, have been used for making microparticles for vaccine delivery. All the polymers that have been used for controlled drug delivery can be used for vaccine delivery (112). 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 from water-soluble polymers frequently requires cross-linking reaction to make the polymers remain insoluble. It is possible that 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 is preferred because it does not require organic solvents or high temperature. Polymers that have been used in the 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 for long term in much the same way as do alum adjuvants, and this results 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.

The size of microparticles is known to play a critical role in oral immunization. In addition to protecting antigens from acidic and enzymatic degradation in the GI tract, microparticulates 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 size microparticles (113). In addition to the small size, microparticles with more hydrophobic surface property are absorbed better than those with more hydrophilic surface property. There are, however, no definite studies confirming or supporting these assumptions. Once microparticles are placed in the GI tract, adsorption of numerous proteins and polysaccharides present in the GI tract would alter the surface chemistry drastically, and it is difficult to correlate a particular surface chemistry of the native microparticles with the absorption ability.

*Virus-like particles* (VLPs) consist of one or more viral-coat proteins. They are very immunogenic molecules that allow for covalent coupling of the epitopes of interest (114). Recently, 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 to induce strong antibody, helper T-cell, and cytotoxic T-lymphocyte responses (114). Parenteral administration of recombinant VLPs of papillomavirus induced VLP-specific humoral and cellular immune responses (115). Immunization of VLPs without adjuvant via mucosal route is also known to elicit specific antibody at mucosal surfaces and also systemic VLP epitope-specific T-cell responses (115).

*Liposomes* are vesicles composed of naturally occurring or synthetic phopholipids. The bilayer structure can be single- or multicompartment. The size can also vary from smaller than 1 μm to larger than 10 μm. When negatively charged lipid molecules, which form liposomes, interact with divalent cations, a solid, multilayered, crystallaine 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 an effective delivery system for oral vaccination.

Liposomes, like other vaccine-delivery systems, can exert immunoadjuvant effects. 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 (116). Studies showed that the positively charged liposomes delivered proteinaceous antigens efficiently into the cytoplasm of the macrophages/antigen-presenting cells where the antigens are

processed to be presented by class I MHC molecules to induce the cell-mediated immune response (116).

Liposomes containing highly immunogenic glyco-proteins of the Sendai virus on their surface, which are called fusogenic liposomes, showed enhanced antigen-specific humoral immunity in mice. The levels of antiovalbumin antibody were markedly increased in serum from mice immunized with OVA encapsulated in fusogenic liposomes. It appears that the fusogenic liposomes function as an immunoadjuvant in inducing antigen-specific antibody production (117).

*Virosomes* are liposomes containing viral fusion proteins that allow efficient entering 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 (118). 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 (119). Other studies have suggested that immunopotentiating reconstituted influenza virosomes can be a suitable delivery system for synthetic peptide vaccines. The virosomes have a great potential for the design of combined vaccines targeted against multiple antigens and multiple pathogens (120).

*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/nonaquous interfaces, reducing surface tension, increasing miscibility, and stabilizing emulsions. Poly-meric micelles made of block copolymers, such as poly(ethylene oxide)-poly(propylene oxide)-poly(ethy-lene oxide), have been used as a delivery system for hydrophobic drugs. They can also encapsulate antigens for vaccination.

*Niosomes* are nonionic surfactant vesicles. They have been used to develop a vaccine-delivery system by peroral and oral routes. 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 (121).

*Cross-linked protein crystals* have been used 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 over an almost 6-month study (122). It is likely that the cross-linked protein crystals release antigen in a slow-release manner, and in this sense, the cross-linked protein crystals function as a depot. The cross-linked protein crystals present high stability, purity, biodegradability, and ease of manufacturing, all of which are highly attractive features for vaccine formulation (122). Because the cross-linked protein crystals are microparticulates, they can also be used for vaccination through various routes.

## IMMUNOMODULATION

Immunomodulation refers to treatments that alter immune responsiveness in a nonantigen-specific manner. Enhance-ment of the immune response is desired in the treatment of chronic infectious diseases and neoplastic diseases, whereas suppression is needed in cases of inappropriate or exaggerated immune response, including allergies and autoimmune diseases. There are numerous treatments that affect the activity of the immune system. The effect of currently available immunosuppressive drugs is very broad, giving these drugs undesirable side effects. The aim of the research in this area is to design treatments that selectively enhance or suppress immune responses. Some of the newer treatment options are those that target costimulatory molecules, and the use of CpG DNA, and cytokines.

### Costimulation

Activation of T-cells requires two signals. The first signal is provided by recognition of MHC/peptide complex by the T-cell receptor. This does not result in proliferation and differentiation of the T-cell unless the T-cell receives a second, costimulatory signal. Several costimulatory signals have been identified, but the major costimulatory signal appears to result from the binding of CD28 on T-cells to B7 molecules on antigen-presenting cells. There are at least two B7 molecules, B7-1 (CD80) and B7-2 (CD86). Activation of antigen-presenting 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) that, other than its name implies, is rapidly expressed on both CD4^{+} and CD8^{+} T-cells after binding of the T-cell receptor to the MHC/peptide complex on antigen-presenting cells. However, in contrast to the positive signal provided by CD28, CTLA-4 downregulates T-cell responses (123). CTLA-4 has a higher affinity for the B7-molecules than does CD28 and may prevent the activation of T-cells when B7 expression by dendritic cells is low and terminate

the immune response when its expression is strongly increased. A soluble chimeric protein, CTLA4Ig, blocks the binding of both CD28 and CTLA-4 to the B7 molecules and, thus, may prevent T-cell activation. Administration of this protein to patients with psoriasis vulgaris, an immune-mediated skin disease, in a phase I clinical trial resulted in significant improvement in approximately 50% of the patients (124). 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 (125).

Other CD28 and B7 homologs continue to be identified and appear to play a role in costimulation (126). 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

Bacterial DNA has a higher content of the CpG dinucleotide than does vertebrate DNA, and, in contrast to vertebrate DNA, the CpG is not preferentially methylated. The unmethylated CpG DNA sequences provides a strong stimulus for the immune system (127). CpG DNA stimulates the secretion of IL-12 by macrophages and dendritic cells and thus provides a potent stimulus for type 1 immune responses. It also directly stimulates B cells to proliferate and differentiate into immunoglobulin secreting cells. A cellular receptor for CpG DNA has not been identified. 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 various signaling pathways.

Applications for oligonucleotides containing unmethylated CpG sequences (CpG–ODN) are being explored in various areas of immunotherapy. Administration of CpG–ODN to mice protected against subsequent challenge with the intracellular bacteria *Listeria monocytogenes* and the intracellular protozoa *Leishmania 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

responses. CpG DNA may also have a place in 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 critical role in the regulation of the immune and inflammatory response, and they are potential targets for therapy. Important limitations, however, are 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 inhibitors.

### Interferon-β

Clinical trials have demonstrated that subcutaneous injections of recombinant or natural interferon-β reduces the rate of exacerbation of relapsing-remitting multiple sclerosis (128, 129). The mode of action of interferon-β has not been determined. Interferon-β reduces the production of tumor necrosis factor-α and increases the secretion of IL-10 in vitro. 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.

### Tumor necrosis factor-α inhibitors

Tumor necrosis factor-α (TNF-α) is a cytokine with multiple biological effects. It 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 the TNFR II that are expressed on many different types of cells. The 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 (130, 131). 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 does the naturally occurring soluble TNFR.

## CHALLENGES IN FUTURE VACCINE FORMULATIONS

Recent advancements in microbial pathogenesis, immunology, genetic engineering, plant genetics, and expression vector technology have formed the foundation for a new generation of vaccines and other pharmaceutical products. New developments in the delivery system have provided us with novel ways to enhance the immunogenicity of subunit antigens or nucleic acids by their controlled release and reduced degradation.

For more convenient and more effective immunization, current vaccine-delivery technologies need to be improved. Currently, vaccination of many inactivated or subunit antigens requires booster doses because of the lack of inherent immunogenicity found in the natural organism. Thus, reducing the number of doses is one of the primary goals in vaccination. 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 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. The requirements and problems of immunizing immunocompromised, immature, older, or pregnant hosts need to be addressed effectively. Further improvement in our understanding of how to modulate Th1 and Th2 responses effectively would certainly help us design better vaccines. Another means of improvement is to combine a number of vaccines into multivalent vaccines. This will improve the

immunization compliance in people living in developed or developing countries. 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. The new-generation vaccine formulations should also have high stability, thus avoiding the problems commonly observed during storage.

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